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BIOLOGICAL BULLETIN

SELECTIVE FERTILIZATION IN POLLEN MIXTURES.

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Equality in fertilizing power of gametes dissimilar in the hereditary factors they carry is a corollary of Mendelism. It has been thought that certain exceptions to this general rule may exist. Differential fecundating ability has been suggested from time to time as a possible interpretation of otherwise unaccountable results. The earlier uses of this hypothesis of inequality in sperm efficiency, such as Castle's (1903) theory of sex determination, Cuenot's (1908) conception of the reason for the non-appearance of homozygous yellow mice, have since been found to be unnecessary. The interpretation of linkage phenomena according to the reduplication hypothesis, while essentially different in application, holds somewhat the same implication as selective fertilization, that is, differential operative power of cells of unlike germinal construction.

As the yellow mouse problem was finally resolved to a selective elimination of zygotes so many puzzles have had considerable light thrown on them by a more complete understanding of the factor relations. Many instances of the non-appearance of certain types are known to be the result of the action of lethal factors. Good illustrations of this are found in Drosophila (Muller, 1918). Other cases of elimination immediately after fertilization or early in development are known in maize.

Sometimes abortion of a part of the gametes before fertilization takes place and it is assumed that the elimination is selective. Belling (1914) has shown that in Stizolobium 50 per cent. of the pollen grains and ovules regularly abort in certain types. The aberrant results from the Œnotheras are now generally considered largely, if not wholly, to be due to differential destruction of both

gametes and zygotes. Davis (1915–'17) has called attention to the great amount of pollen and ovule abortion in these plants and to the low germination of the seeds which are produced and to the results which are obtained when a more complete germination is secured.

It is not always easy to distinguish between differential destruction of zygotes immediately after fertilization and selective fertilization. It is still more difficult in the case of abortion of germ cells. In fact it is only a matter of degree between selective elimination of gametes and selective fertilizing ability. From germ cells which are unable to complete development on account of the particular inheritances they carry, to gametes which appear normal but are unable to function perfectly under any circumstances, it is only a step. However, no instance of the latter condition is positively known and the cases of gametic abortion clearly due to the factors which the gametophytes carry are rare.

Although results still remain to be cleared up which indicate a selective action of some kind (Kempton, 1919) the general conclusion holds, which is, that the pollen, although gametophytic itself, has the function of the sporophyte which produces it. This is supported by the well-known cases where pollen color and shape are all of the type of the maternal parent irrespective of the factors for pollen characters which they carry. Many instances are known where gametes, containing lethal factors which stop development immediately after fertilization, are able to function fully as well as others permitting normal growth. East and Park (1917) have shown that compatibility in Nicotiana is alike for all the gametes of any one individual. Different pollen grains may carry factors which will determine sterility or fertility of the future zygotes but all function alike in fertilization according to the sporophyte from which they come. Following up these results East (1919) shows that in the frequency distributions of pollen tube lengths there is no significant difference between pollen from plants greatly unlike in the degree of heterozogosity. In other words gametes carrying markedly diverse germinal complexes are no more variable than those all of like constitution in ability to send pollen tubes down the styles

and to accomplish fertilization. East considers that chance segregation in the germ cells and random mating of these germ cells is a fundamental genetic hypothesis applicable to plants and animals alike. With this conclusion the writer is in accord, with the reservation that the evidence from gametic abortion should not be put aside as belonging to a different category.

The data to be presented here bear upon another phase of the problem. The above conclusion, it should be clearly kept in mind, applies only to gametes produced by one or more individuals of the same type. That is, the gametes may be unlike in the factors they carry, but if they come from the same or similar individuals they are potentially equal in ability to fertilize. But what is the result when germ cells from two individuals of different type are presented at the same time in excess so that not all can fulfill their function? Will fertilization take place at random or not? This is the problem to be considered here. In the one case doses of different kinds of medicine come in the same capsules. In the other the capsules as well as their contents may differ. Anyone accustomed to swallowing a particular kind of capsule made of a familiar substance and all of the same size and shape can take any kind of medicine with equal readiness irrespective of the result which will ensue when the materials within the containers begin to operate. Applying this crude metaphor to plants and animals, in the one case, the germ cells, however they differ in factorial composition, come in the same cytoplasmic envelope; in the other, the cytoplasm as well as the genes may differ.

It has already been shown that, in particular cases, there is a selective action when pollen from different plants is applied to a stigma at the same time. East (1919) has demonstrated that in a mixture of compatible and incompatible pollen placed on the stigmas of self-sterile Nicotianas the compatible pollen alone functions. Two experiments were devised to test this. Small numbers of pollen grains were counted out under a microscope and placed on stigmas known to be receptive to that kind of pollen. The stigmas were then covered with a large amount of incompatible pollen. The former only was able to fertilize as shown by the results from eight such mixed pollinations which

in no case produced a greater number of seeds than the number of compatible pollen grains applied. In the other experiment a constant white-flowered self-sterile plant was pollinated with its own pollen. Several hours later pollen of a red-flowered plant was applied. Abundant seed was secured and when grown red-flowered cross-fertilized plants only were found. In both these cases there was complete selective fertilization in favor of the pollen from dissimilar plants.

According to the results to be reported here a directly reversed effect is obtained from mixed pollinations in maize. In many trials the plant's own pollen has been more efficient in accomplishing fertilization than that from other individuals which differ only in minor features. This same pollen which is less effective when in competition with the plant's own kind of pollen is fully able to function when not applied in mixtures. The results are remarkable in view of the notable advantages which cross-fertilization gives to the immediately resulting seeds and the plants grown from them. The material used consisted largely of self-fertilized strains which had been brought to uniformity and constancy and were considerably reduced in size and vigor. In this material crossing is known to increase the weight of seed within the same inflorescences as much as 50 per cent. in some cases. This greater amount of material is laid down both in the embryo and endosperm, and is apparent in the greater size of the seeds which show a higher specific gravity together with more rapid maturation indicated by their lower water content at the close of the growing period. A large series of mixed pollinations show the ability of the cross-fertilized seeds to germinate better by an average of 16 per cent. The resulting plants start to grow sooner, develop faster, mature in a shorter time and at the end far surpass their self-pollinated sibs. The amount of heterosis shown by maize is possibly greater than that displayed by any other plants in intra-specific crosses. Production of grain has been advanced on an average of 180 per cent., height of plant 27, length of pistillate spike 29, number of rows of spikelets 5 and number of nodes 6 per cent. in a study of a large number of crosses between inbred strains (Jones, 1918).

In spite of these great immediate advantages to be secured the

plants manifest a decided preference for their own kind of pollen. This is a result which would have surprised students of flower pollination in Darwin's time but which, as I shall attempt to point out later, is in agreement with other results from biological investigation.

Methods of Carrying on the Experiments.

In an investigation in which it was desired to compare the chemical composition of seeds of maize having different genetic constitution but produced under as nearly identical conditions as possible advantage was taken of endosperm characters to enable proper classification of seeds produced in the same inflorescences. For example, two kinds of pollen carrying yellow endosperm color and white endosperm color were mixed together and applied to a plant which normally produces uncolored seeds. The resulting yellow and white seeds were distributed at random on the pistillate spikes and were as nearly comparable in respect to external and nutritional factors as it is possible to obtain. It was found that such pollen mixtures could be applied to both strains furnishing the pollen and the two kinds of seeds easily distinguished. On the white-seeded strain the seeds resulting from the plant's own pollen were white and the cross-fertilized seeds yellow. On the yellow-seeded plants the self-pollinated endosperms were dark yellow while the crossed seeds were distinctly lighter in color, in most cases they had a white cap, and were as a rule readily separable.

Attention is directed to the fact that the material used for these mixed pollinations consisted largely of inbred strains which had been reduced to uniformity and constancy so that the genetical differences between the two kinds of seeds sharply differentiated them, much more than in ordinary cross-pollinated varieties of this plant in which the yellow color is usually variable due to more than one factor for this color and various modifying conditions such as the consistency of the endosperm in respect to corneous and floury starch.

A number of pairs of plants were treated in this way by mixing their pollen and applying to both types. After harvesting it was realized that here was an excellent method of determining

whether or not any selective action was shown by the plant's own pollen as compared to that from a plant of somewhat different type. If one member of the pair of plants which furnished the pollen for the mixture is designated A and the other B, the two kinds of seeds grown on A plants are A × A, self-fertilized, and A × B, cross-fertilized; on the B plants B × A cross-fertilized and B × B self-fertilized. Since the same pollen mixture is applied to both, the ratio of the seeds resulting from A pollen to the seeds resulting from B pollen, on A plants, should be the same as the ratio of the seeds resulting from A and from B pollen on B plants. In other words, the numbers form a proportion which, irrespective of the relative amounts of A and B pollen in the mixture, should be a perfect proportion within the limits of random sampling if fertilization takes place at random. The end terms of the proportion comprise the self-pollinated seeds and the middle terms the reciprocally cross-pollinated seeds. If a true proportion is obtained the products of the end terms should naturally equal the products of the middle terms. If they do not the deviation is either in an excess of cross-fertilized or of self-fertilized seeds indicating a selective action in one or the other direction.

The advantages of this method of attacking the problem are readily apparent. It is practically impossible to make up a mixture of large amounts of pollen in which the proportion of each is known. Either measuring or weighing the pollen is out of the question because maize pollen takes up moisture from the air rapidly and when any quantity is brought together it becomes aggregated into a flocculent mass. Moreover this pollen soon loses its viability so that even in case equal numbers of pollen grains could be had there would be no proof that there were equal numbers of functional pollen grains in the mixture.

The method of reciprocal application and arranging the results in the form of a proportion automatically overcomes all these difficulties and the experiment is as simple as could be devised. An attempt was made to have as nearly equal quantities of pollen as possible by measuring out the two kinds roughly. But in many cases the results showed that one kind of pollen was far more effective than the other. This, however, does not destroy

the value of the figures as it is the relative efficiency of each type of pollen, when it is applied to its own and to foreign stigmas, that is being investigated.

In the preliminary experiments there were 18 pairs of plants which produced seed as the result of the application of mixed pollen. When these were counted and the deviations of the proportions found from the closest perfect proportions were obtained there were 12 mixtures which showed a deviation in favor of the self-fertilized seeds and 6 in the opposite direction. The results as a whole showed a tendency to favor the plant's own pollen. This was somewhat unexpected so that it was considered worth while to investigate the matter more fully.

The inbred material was so reduced in growth that the number of seeds produced on one plant was not large enough to give the results much weight. It was therefore decided to pollinate a number of plants of two different self-fertilized strains with the same mixture. Most of the strains used had been self-pollinated for six generations or more, some as many as ten, so that the plants within one strain were practically identical in hereditary constitution. Pollen was collected from about the same number of plants as the pollen was applied to. The two lots of pollen were put together in a paper sack and thoroughly mixed by shaking. This mixture was then applied to plants of the two strains which supplied the pollen. It was desired to have from ten to fifteen plants in each of the paired strains so as to give from 1,000 to 2,000 seeds in each of the two parts of the proportions but the flowers were not always ready at the right time and some pollinations were failures for a variety of reasons so that not as large numbers as desired were secured in every case.

Every effort was made to prevent the entrance of undesired pollen. The technique has been described and the amount of experimental error due to contamination to be expected in artificial pollination of maize has been considered previously (East and Hayes, 1911). In the course of these experiments the effects of extraneous pollen were seen in very small numbers compared to the total number. This source of error could be detected in the seeds when colors or other characters differing from either of the strains used were brought in by the undesired

pollen. In a total of 63,000 seeds produced in these experiments only about 30 illegitimate seeds were observed. A larger number of contaminations, however, would have the same appearance as the legitimate seeds and so could not be detected but giving a reasonable allowance to this source of unreliability the results could not be appreciably altered. Moreover the error of this kind is never all in one direction so that in the main it can be disregarded.

The accuracy with which the seeds have been classified deserves particular attention. Strains were selected to be used which gave sharp differences between self-fertilized and cross-fertilized seeds and in most cases separation was made very satisfactorily. In a few mixtures there was some doubt and in two experiments the seeds on the yellow endosperm plants could not be distinguished. In these two cases the seeds were planted and classification was made with the mature plants. Also in all the other mixtures involving yellow and white endosperms a sample was taken, after all the seeds of one class were mixed together, and grown to determine the per cent. of error in separating the seeds. Since the self-fertilized seeds give small inbred plants, pure for yellow or white color, while the cross-fertilized seeds produce large vigorous hybrids segregating into yellow and white seeds, classification of the mature plants can be made without the least doubt. However, it should be noted that the better germination and greater vigor give the advantage to the cross-fertilized classes in every case if there is any difference. About 120 plants in each lot were grown and the per cent. of error obtained was used to calculate the total amount of error if all the seeds had been grown. Since the numbers of seed ran up into the thousands it was impossible to grow all of them. The figures showing the per cent. of errors found are arranged in Table III. Only in one case is the number of wrongly classified individuals above 3 per cent. In 25 out of 44 lots no faulty separations were discovered. In the remaining cases the misplaced seeds tend to balance each other so that this source of doubt can be largely removed. Calculating the figures without regard to the error of classification gives practically the same results as when this is taken into consideration.

Different characters were used in other mixed pollinations which permitted even more positive classification than the single character difference of yellow and white endosperms. Plants with yellow sweet seeds were paired with white starchy plants. Each strain brought in a dominant character. White starchy plants of the pop or *Zea mays everta* type were used because the clear corneous endosperm differentiates very clearly between yellow and white. In the reciprocal cross the smooth opaque seeds show up plainly contrasted with the wrinkled translucent seeds. Similarly purple sweet and white starchy types were paired. In a few cases the purple crossed seeds were not as distinct as could be desired, but the error is certainly small. These latter mixtures were made the past season and no plants have been grown to test the reliability of their separation but the writer is confident that the number of wrongly classified seeds, if any, is not sufficient to alter the results appreciably.

In many inflorescences a few seeds were found which had failed to reach a stage of development so that they could be classified. Seeds at the tips of the spikes and where the seeds were closely crowded were abortive. This introduces another source of unreliability, that of selective elimination of zygotes. Since crossing gives to seeds of maize an enormous advantage in development, it can confidently be expected that as a rule more of the self-pollinated seeds will be found among the abortions than cross-pollinated. What we are attempting to study is the relative fertilizing efficiency of different types of pollen grains. But one can only arrive at this by counting the zygotes sometime after fertilization has taken place. In the meantime a differential destruction of zygotes may have taken place. This effect must be considered in any organisms employed but because of the short time which elapses between fertilization and the maturation of the seeds, and from the fact that they develop in an exceedingly favorable and uniform environment, maize is the very best material the writer can think of in which this problem can be attacked, especially when the numbers which can be obtained are taken into consideration. All animals and those plants which do not show zenia in the seeds have the objection that a comparatively long time elapses between fertilization and suffi-

cient maturity to permit classification. In plants many cases are known in which there is a selective elimination of certain classes of individuals due to a lesser germination and unequal ability to grow. In Drosophila (Hyde, 1914) crossing does not influence the number of eggs laid but markedly regulates the per cent. that hatch. Therefore the error from this source always tends to show an apparent deviation in favor of cross-fertilization. In maize where the seed progenies can be used this differentiating effect is at a minimum and probably is not sufficient to affect the numbers appreciably but since the tendency is in the opposite direction to the results which have been obtained the data are even more convincing.

TABLE I.

CHARACTERS OF THE SEEDS AND PEDIGREE NUMBERS OF THE INBRED STRAINS AND FIRST GENERATION HYBRIDS USED IN THE MIXED POLLINATIONS.

Pollen Mixture Number.	Characters of the Seeds of		Pedigree Number of	
	A Strain.	B. Strain.	A Strain.	B. Strain.
1.....	Yellow Starchy	White Starchy	1-9-1-2-4-6-7-5-6-2-1	10-4-8-3-5-3-4-8-2-1
2.....	" "	" "	1-9-1-2-4-6-7-5-3-2-1	10-4-8-3-5-3-4-5-2-1
3.....	" "	" "	14-4-6-16-2-12-22	21-13-9-7-57-11
4.....	" "	" "	14-4-6-16-2-12-22	21-13-9-7-57-11
5.....	" "	" "	14-4-6-16-2-12-22	21-13-9-7-57-11
6.....	" "	" "	14-30-4-3-7-11-10	21-13-9-7-57-11
7.....	" "	" "	14-30-4-3-7-11-10	20-4-25-47-4
8.....	" "	" "	14-4-6-16-2-12-22	20-8-5-35-20
9.....	" "	" "	1-9-1-2-4-6-7-5-6-2-1-1	20-4-25-47-4
10.....	" "	" "	(1-6-1-3 × 1-9-1-2) F_1	(20-8 × 21-13) F_1
11.....	" "	" "	(1-6-1-3 × 1-7-1-1) F_1	(20-8 × 21-13) F_1
12.....	" "	" "	(1-7-1-2 × 1-6-1-3) F_1	(21-13 × 20-8) F_1
13.....	Yellow Sweet	White Starchy	146-1-1	65-8-2-2-6-5-3-2-1-1-1
14.....	" "	" "	146-1-1	65-8-2-2-6-5-3-2-1-1-1
15.....	Yellow and White Sweet	" "	(126-1-1-1 × 77) F_1	117-3-1-1
16.....	" " " "	" "	(126-1-1-1 × 77) F_1	117-1-1-1
17.....	Purple Sweet	White Starchy	76-2-2-1-1	117-1-1-1
18.....	" "	" "	76-2-1-2-1	117-1-1-1
19.....	" "	" "	76-2-1-2-1	20-4-25-47-24-1
20.....	" "	" "	76-2-2-1-1	20-4-25-47-24-1

	C Strain	C Strain
1.....	Yellow Starchy	1-6-1-3-4-4-4-2-4-4-2-5
19.....	White Sweet	77-2-1-1-1
20.....	White Starchy	117-1-1-1

Materials Used and Presentation of the Results.

The figures obtained from the preliminary experiments in which pairs of single plants only were used are not given here because the numbers are too low to give the results much value and for fear of making this report too bulky. It is sufficient to remember that the data taken together indicated a slight selective action favoring the plant's own pollen.

Table II.

Summary of All the Pollen Mixtures giving the Total Number of Seeds, the Numbers in Each Class forming Proportions, the Deviations of which, Expressed as per cent., from the Closest True Proportions, are Almost Wholly in Favor of the Plant's Own Pollen. P is the Probability that these Deviations are Due to the Differences of Random Sampling.

Pollen Mixture Number.	Total Number of Seeds.	A×A.	A×B.	B×A.	B×B.	Deviation from True Proportion, Per Cent.	χ^2.	P.
1	3,430	1,738	46	1,602	44	+ .045	.027	.994
2	5,636	2,133	145	3,080	278	+ .955	7.572	.063
3	1,362	229	14	770	349	+12.715	146.196	.000
4	3,344	710	126	1,856	652	+ 5.465	61.203	.000
5	1,956	589	6	1,290	71	+ 2.105	28.718	.000
6	424	40	71	187	126	−11.850	23.858	.000
7	3,459	23	89	1,507	1,840	−12.245	235.357	.000
8	7,783	2,185	956	2,619	2,023	+ 6.570	144.108	.000
9	8,729	2,550	1,288	2,922	1,969	+ 3.350	42.061	.000
10	5,408	1,084	1,154	997	2,173	+ 8.495	162.687	.000
11	3,314	448	264	1,505	1,097	+ 2.540	8.937	.030
12	3,561	1,724	719	749	369	+ 1.790	5.313	.053
13	736	185	391	95	65	−13.625	55.051	.000
14	792	424	150	156	62	+ 1.155	.533	.894
15	3,168	2,609	47	14	498	+47.750	2,889.561	.000
16	2,224	723	8	74	1,419	+46.975	1,965.981	.000
17	1,410	1,303	3	4	100	+47.960	1,298.993	.000
18	1,599	4	21	1	1,573	+ 7.970	137.532	.000
19	2,606	528	392	343	1,343	+18.525	376.394	.000
20	2,753	897	77	1,174	605	+13.050	283.002	.000
	63,694							

Of those experiments in which pollen was applied to several plants of the same type 20 pollen mixtures in all have been made.

Each mixture is given a number and the seed characters of the materials used and their pedigree numbers are given in Table I. The first number in the pedigree designates the variety from which the inbred strains were derived. The following numbers indicate the progenitors in the successive self-fertilized generations. The total number of units in the pedigree number less one, show the number of generations the material had been self-fertilized at the time the pollinations were made. The inbred strains used in these experiments are as follows:

1. Several distinct strains from a yellow dent variety originally obtained in Illinois and known as Chester's Leaming, self-fertilized ten or more years.

10. A strain with white floury seeds with no traces of corneous starch, self-fertilized nine generations.

14. Two distinct strains from a yellow dent variety from Connecticut known as Stadtmueller's Leaming, selected for high protein content during six generations of self-fertilization.

20. Two distinct strains from a white dent variety originally selected for high protein at the Illinois Exper. Station and further selected during four generations of self-fertilization.

21. One strain from same source as above but selected for low protein in field pollinated cultures and during five generations of self-fertilization.

65. A small, white, round-seeded strain from a variety of popcorn, *Zea mays everta*, self-fertilized ten times and characterized by clear corneous starch.

76. Two similar strains from a sweet variety of latent flint type having purple aleurone and known as Black Mexican, self-fertilized four years.

77. From a sweet variety of latent dent type with deeply wrinkled white seeds known as Evergreen and self-fertilized four generations.

126. From a small, early maturing, yellow, sweet variety of latent flint type with dark yellow kernels, known as Golden Bantam, and self-fertilized three generations.

117. From a variety of popcorn with sharp pointed seeds having clear corneous endosperm, self-fertilized three times.

146. From a yellow, sweet variety, Golden Bantam, of different

source than 126, and somewhat different in type, self-fertilized two years.

Mixtures number 1 to 9 inclusive comprise various inbred strains with yellow starchy and white starchy endosperm. Some of these strains have been described previously (Jones, 1918) and all show marked heterosis in the crossed seeds and in the resulting first generation hybrid plants. A sample of all the different lots of seed secured in these mixtures have been grown to test the accuracy of classification. Pollen mixtures number 10 to 12 are not from inbred strains but from first generation hybrids, one having all yellow seeds the other all white. They were of such a constitution that the second crossing gave still more increase in vigor although not as great as the stimulus following the first cross. The plants being vigorous a large amount of seed was obtained from a few plants. It was desired to know whether the same selective action would be shown by vigorous plants with segregating gametes as contrasted with non-vigorous plants whose gametes were all alike. Classification of the seed was easily carried out and the per cent. of error when tested was found to be quite low. Mixtures 13 and 14 involved yellow sweet in one strain and white starchy endosperm in the other. One dominant factor was carried by each so that differentiation was perfectly distinct in the reciprocal applications. In mixtures 15 and 16 it was intended to make use of the same characters as in the two preceding numbers. The plants which were supposed to be inbred individuals of a yellow sweet strain, and so labeled, when grown in the field were seen to be too vigorous for this material as it had behaved in previous years. The plants were all alike, however, in this respect. It was suspected that this was a lot of first generation hybrids instead of plants from self-fertilized seed. As some crosses had been made with plants of this line the previous season it is now certain that in this instance the seed was not properly labeled at harvest and so was planted for self-fertilized seed when in reality it was all cross-fertilized. As no other plants were available at the time they were needed these were used. Several self-pollinations were made at the same time to show what the seed characters were of this undoubted hybrid. At maturity it definitely showed itself

to be a hybrid as all the ears were segregating for yellow and white sweet seeds. It therefore had certainly been crossed with a white sweet inbred strain the year before as other pollinations of this sort were made at that time. The yellow color of the endosperm was not sufficiently diluted to cause the seed to be suspicioned before planting.

Since the plants were segregating for yellow and white it was not material which would have been used ordinarily for mixing with a white starchy strain. The effects of the starchy-carrying pollen showed up all right among the all sweet seeds but the reciprocal cross-pollination showed only the yellow cross-fertilized seeds. The white cross-fertilized seeds of course could not be distinguished from the self-fertilized seeds. But since half of the pollen grains carried yellow and half of them white the number of yellow seeds can be doubled to give the total number of cross-pollinated seeds and the assumed number of white cross-fertilized seeds subtracted from the white seeds. This increases the error from random sampling somewhat but since the number of yellow seeds is very low in comparison with the white in these mixtures, the data are reliable in view of the great selective action shown in these two mixtures. The fact that the yellow color in this material was a unit factor difference and that there were equal number of pollen grains carrying yellow and white is proven by the self-fertilized ears produced by the hybrid which gave very good 3 to 1 ratios (one ear counted gave 318 yellow and 97 white seeds). Furthermore the starchy crossed seeds produced on the hybrid plants were of two kinds, yellow and white, and were produced in equal numbers (actual numbers: 28 yellow and 27 white. Since the ovules were segregating equally and the self-fertilized seeds gave the mono-hybrid ratio the pollen grains must have carried the two colors in equal amount.

In the last four mixtures the characters, purple sweet and white starchy, were used. In numbers 17 and 18 the plants were not producitve and the numbers of seeds are low. Also the classification of purple starchy cross-fertilized seeds and white starchy self-fertilized was not as sure as in the other mixtures. In the last two mixtures satisfactory numbers were obtained and the differentiation was clear-cut on both sides.

The pollen mixtures 1, 19 and 20 were also applied to a third strain distinct from either of the two used in supplying the pollen. The resulting two lots of seed in each case were both cross-fertilized and probably showed hybrid vigor in the seed in about equal amount. Heterosis is clearly apparent in the plants of similar crosses involving the same or closely related material and is approximately equal.

The data from each pollen mixture are presented in the form of an appendix. Since the arrangement is the same in all the tables these are given in the simplest form possible and a description of one applies to all. In the tables the headings for the four different classes of seed are A × A, A × B, B × A, B × B and in the three mixtures 1, 19 and 20 there are in addition the out-crossed seeds C × A, and C × B. In every case the pistillate parent is given first. These headings when expanded in detail are as follows:

A × A = Self-fertilized seeds from color-carrying pollen.
A × B = Cross " " " non-color " "
B × A = Cross " " " color " "
B × B = Self " " " non-color " "
C × A = Out-crossed " " color " "
C × A = Out " " " non-color " "

On every plant there are two kinds of seeds indicating the relative fertilizing efficiency of the two kinds of pollen in the mixture used. The number of seeds from individual plants are given in the tables under their respective headings, A × A and A × B seed from the same inflorescence and B × A and B × B seeds from another and C × A and C × B from a third. The total number of seeds is summed up below the line. Next to that is given the per cent. of error found upon testing the accuracy of the classification. This figure is used to calculate the amount of error based upon all the seeds obtained. Not more than about 125 seeds were grown in each lot except in two cases as shown in Table III. The calculated amount of wrongly classified seeds is given on the line below the per cent. of error, then follows the corrected numbers after the proper additions and subtractions have been made. After that is placed the observed proportion

of the two kinds of seed in each member of the pair stated as per cent. The closest perfect proportion is calculated from this, based on the results from the A and B plants but not from the C plants, and this subtracted from the actual proportion found gives the deviation in per cent. which appears in the last line. This description applies to mixtures 1 to 12 inclusive. Of the remainder no correction for misplaced seeds is made.

TABLE III.

NUMBER OF PLANTS GROWN AND THE PER CENT. OF WRONGLY CLASSIFIED SEEDS IN THE POLLEN MIXTURES INVOLVING YELLOW AND WHITE, STARCHY ENDOSPERM.

Pollen Number. Mixture	A. × A.		A. × B.		B. × A.		B. × B.	
	Number.	Per Cent.	Number.	Per Cent.	Number.	Per Cent.	Number.	Per Cent.
1.....	414	1.21	26	7.69	107	0	27	22.22
2.....	483	2.69	50	8.00	115	0	128	2.34
3.....	105	0	12	0	119	0	114	1.75
4.....	116	0	101	.99	120	.83	114	1.72
5.....	110	0	6	0	120	0	56	0
6.....	40	—	71	—	112	0	76	2.63
7.....	23	—	89	—	119	0	118	0
8.....	119	0	118	0	120	0	118	1.69
9.....	118	2.54	118	2.54	120	1.67	113	0
10.....	117	1.71	118	0	118	1.69	117	1.71
11.....	114	.88	119	0	123	0	120	0
12.....	116	.86	117	0	116	0	116	0

The total number of seeds, the numbers in each class and the deviations in per cent. from the closest perfect proportion for the 20 pollen mixtures are summarized in Table II. The deviation, if it is an excess of self-fertilized seeds, is given as plus and, if the opposite, as minus. The deviations can range from + 50.0 to − 50.0 per cent. The extremes indicate complete non-functioning of each kind of pollen on one set of plants and exclusive functioning on the other.

Altogether the number of seeds amounts to 63,694. Large numbers are, of course, necessary to be convincing in any experiment on selective fertilization as such investigations are largely studies in sampling. Mixtures number 6, 7, 13, 17 and 18 are less reliable than the others, because they have rather small numbers from either the A or B plants, less than 200 in each case. Like the chain with the weak link the value of each set of figures

is dependent upon the numbers in the less populous half of the proportion.

Some of the mixtures have over one thousand individuals in each of the four classes. Since the experimental error is low and not all in one direction as has been shown and the selective elimination of zygotes tends to obscure the result which has been obtained, such figures as these can not be gainsayed. Of the 20 mixed pollinations 17 show a deviation indicating a selective action in favor of the plants' own pollen. While three of the mixtures show the opposite effect. These three are all low in number of individuals on one or the other side of the proportion. Mixtures 6 and 7 could not be classified by the seeds on the yellow seeded plants so consequently the progenies were grown and classified at maturity. This brings in other sources of error—differential germination and competition between plants which are weak with those that are vigorous—which certainly tend to result to the apparent advantage of cross-fertilization. Two of the deviations showing an excess of self-fertilized individuals are not significant when compared to the allowable differences from random sampling but all the others are. Therefore the conclusion is inevasible that in maize the plant's own pollen is more effective in consummating fertilization than pollen from plants of only slightly different construction. This selective action is shown even though the foreign pollen is perfectly capable of fertilizing the plants when not acting in competition with the plant's own kind of pollen as has been definitely proven. Mixtures number 15, 16 and 17 show 47 out of a possible 50 per cent. deviation, almost complete non-functioning of the unfamiliar pollen. Numbers 15 and 16 include the first generation hybrids in which some calculations had to be made to allow for segregation but these are perfectly justifiable adjustments and the results can be discounted but very little. Mixture 17 is low in numbers having only 104 seeds on the B plants of which 4 are cross-fertilized. But observe the result when this same mixture is applied to the A plants. Here only 3 cross-fertilized seeds are to be found among 1,303 self-fertilized seeds. Surely there is some powerful action working to hold back the unfamiliar pollen. Mixtures number 3, 4, 8, 10, 19 and 20 are by them-

selves convincing as in these the numbers are large, the differentiation of the seeds in both groups is precise and the deviations clearly show the superiority of self-pollination.

The data from pollen mixture number 1 have been published previously (Jones, 1918) although at that time plants had not been grown to test the error in separating the seeds. The greatest number of mistakes of classification of any of the mixtures were made in this lot and the deviation is now well within the limits of random sampling. The data are included here to make this report complete. In the previous publication the probable error used was the familiar formula used for Mendelian ratios. The determinations applied to each half of the proportion alone. Since the ratio on each of the paired plants is dependent upon the ratio on the other, it seems to the writer now that the use of this method of calculating the probable error in connection with this particular problem is wrong.

The method of calculating the significance of the figures as used here is that proposed by Elderton (1901) and is in general use in presenting genetic data. It is obtained in the following manner. The deviations of the terms in the actual proportion found from the closest perfect proportion as calculated are squared and divided by the terms of the perfect proportion. Their sum gives a value χ^2, which by use of convenient tables calculated by Elderton, gives a probability value varying from 0 to 1 proportional to the goodness of fit.

The calculations must be based on the actual numbers of seeds obtained and not on the percentages. To obtain a perfect proportion from which the deviations of the numbers found will be the smallest in the four terms it is necessary to balance the figures so that the same number of individuals are represented on the A and B plants. This is done by reducing the number of seeds of the greater and increasing the lesser keeping the ratios the same, of course. The deviations of the proportions, balanced in this way, from the closest perfect proportions are then used to obtain the probability value in the way described above. The same result is obtained more quickly by calculating the value of χ^2 from the percentages and multiplying this figure by one half the total number of seeds.

With four terms the values of χ^2 greater than 18 have no probability out to three decimal places. Since very much greater numbers were secured in most cases, as shown in Table II., the deviations are clearly not due to the differences of random sampling alone if the application of this method of calculation is justifiable. The writer is not perfectly sure that it is because it should be noted that the theoretical proportion is calculated directly from the results found, that is, there is no possible way of knowing the real amounts of the two kinds of functional pollen contained in the mixtures. Moreover the probable error does not take into consideration the corrections which are made for the mistakes of classification found in a sample drawn from each lot.

There may be a selective action when the pollen is applied to one plant but not to the other or the action may be reversed. All that is measured is the combined effect if both are in the same direction or the excess of one over the other if in opposite directions. It seems reasonable to suppose, however, that the selective fertilization is approximately the same on both members of the pair as a large number of mixed pollinations are avai able made with many different types of plants and the majority give the same result. If this were always true, however, the ratio obtained by out-crossing the mixture onto a distinct strain should not deviate from the ratio of the closest perfect proportion calculated from the figures of the reciprocal crosses beyond allowable limits. That is, the ratio obtained from the out-crossed seeds is supposed to represent very nearly the actual ratio of effective pollen in the mixture since both kinds are more nearly on the same footing. In the three experiments, Nos. 1, 19 and 20 in which such out-crossed seeds were obtained, the deviation is even greater in two cases than that from the reciprocal applications. In pollen mixture number 1 all the deviations are small and probably without significance.

Since the results are convincing when considered without a probable error, it is not necessary to lay much stress on the method of its calculation at this time. Considering the data altogether, magnifying the actual experimental error to its fullest extent, and taking a common-sense view of the allowances to be

made for variations inherent in a problem of this kind the conclusion can be no other than that these plants manifest a definite receptiveness to their own pollen, discriminating against foreign pollen even though it comes from plants only slightly differentiated from them, both of which might easily be descended from the same individual at no very distant period back. This selective action is shown by plants of weak growth or full vigor, whether each strain descended from a line of similar ancestors or whether its immediate parents were diverse and, finally, irrespective of the gametes being alike or unlike in germinal contents. The one significant feature in common in all these experiments is the fact that the cytoplasm which surrounds the male nuclei and which makes up the vehicle that carries them to the egg cells is alike for the gametes of any one type of plant whether this plant is homozygous or heterozygous and in self-fertilization this cytoplasm is the same as the medium in which the pollen fulfills its function. This points very strongly to the probability that the differential effect is due to the rate of pollen-tube growth although it may be determined after the male nuclei are brought to the egg.

The average weight of the seeds in the different classes of all the pollen mixtures is given in Table IV, with the increase in weight of the cross-fertilized over the self-fertilized seeds. Expressed as per cent. these figures permit an estimation of the comparative amount of heterosis shown in the crosses. In fact this is one of the best means of measuring the stimulation of heterozygosis as the environmental differences are reduced to a minimum. It has not been definitely proven that there exists a correlation between the amount of heterosis in the seeds and that shown by the resulting plants grown from those seeds but the indications are that there is a close relation between the two. Since hybrid vigor is roughly proportional to the germinal differences in the two forms united it can be determined whether or not there is a relation between the diversity of the plants used in the several pollen mixtures and the degree of preference shown by those plants to their own kind of pollen. Table V. shows that the coefficient of correlation between the average increase in weight of seeds and the deviation in favor of self-fertilization, both stated

TABLE IV.

THE AMOUNT OF HETEROSIS SHOWN BY EACH CROSS-POLLINATION IN THE INCREASE IN AVERAGE WEIGHT OF SEEDS COMPARED TO THE SELF-POLLINATED SEEDS GROWN IN THE SAME INFLORESCENCES.

Pollen Mixture Number.	Average Weight of Seeds in Centigrams.						Per Cent. Increase.		
	A × A.	A × B.	Increase.	B × A.	B × B.	Increase.	A.	B.	Ave.
1	13.8	18.1	4.3	18.0	15.7	2.3	30.4	14.6	22.5
2	12.2	17.1	4.9	17.6	14.3	3.3	40.0	23.0	31.5
3	29.3	35.5	6.2	39.8	35.7	4.1	21.2	11.5	16.4
4	26.9	31.4	4.5	34.2	29.3	4.9	16.7	16.7	16.7
5	30.0	47.6[1]	17.6	38.3	34.8	3.5	58.7	10.1	34.4
6	—	—	—	36.7	34.7	2.0	—	5.8	5.8
7	—	—	—	19.6	16.3	3.3	—	20.2	20.2
8	25.8	30.6	4.8	23.8	22.5	1.3	18.6	5.8	12.2
9	12.0	15.8	3.8	15.7	13.2	2.5	31.7	18.9	25.3
10	32.2	33.4	1.2	32.5	30.9	1.6	3.7	5.2	4.5
11	27.5	28.6	1.1	34.5	32.8	1.7	4.0	5.2	4.6
12	32.4	32.8	.4	37.4	34.3	3.1	1.2	9.0	5.1
13	25.7	29.3	3.6	16.4	14.8	1.6	14.0	10.8	12.4
14	20.2	22.1	1.9	13.9	12.9	1.0	9.4	7.8	8.6
15	24.2	35.1	10.9	12.9	11.7	1.2	45.0	10.3	27.7
16	32.0	40.0[1]	8.0	11.4	9.6	1.8	25.0	18.8	21.9
17	14.3	20.0	5.7	15.0	10.2	4.8	39.9	47.1	43.5
18	25.0	23.8[1]	−1.2	10.0[1]	8.2	1.8	−4.8	22.0	8.6
19	15.7[1]	22.2	6.5	16.6[1]	15.6	1.0	41.4	6.4	23.9
20	14.3	17.1	2.8	15.9	15.3	.6	19.6	3.9	11.8

in per cent., is $+.496 \pm .093$. Although the numbers are scanty there is a significant relation between the two. This means that the more unlike the plants are the greater the distinction that is made between the two kinds of pollen. *In proportion as the cross-fertilization benefits the immediate progeny in its development the less effective is that pollen in accomplishing the union.*

The same method of experimentation was applied to another plant, the garden tomato, *Lycopersicum esculentum* Mill. Advantage was taken of plant characters such that the seedlings could be distinguished in both reciprocal applications. Pollen from a variety with entire leaves with a tall habit of growth was mixed with pollen from a dwarf variety with normal, serrate leaves. Tall stature and normal leaves are dominant so that the cross-fertilized and self-fertilized seedlings from one variety were

[1] Number of seeds too few to make averages reliable.

visible because of differences in leaf formation and in the other variety by habit of growth. Dwarf plants are characteristically shorter and more compact in stems and leaves which gives them a distinct appearance. The plants used were grown from unpedigreed seed but the tomato is usually self-fertilized and the varieties employed were tested and found to come true to type. Two experiments were made and the results from these are given as pollen mixtures number 21 and 22 in the appendix. The plants from individual fruits are grown separately. The total numbers in the two mixtures are 340 and 272. Differentiation was sharp in the seedling stage in the A lots with either serrate or entire leaves. In the B lots the presence of tall and dwarf seedlings could be easily seen but not all of them could be separated as positively as could be desired. The plants were therefore set in the field and classified after they had grown about two months. They were set out too late to make a satisfactory growth and even at the end of the season classification was not made with certainty in every case.

TABLE V.

CORRELATION BETWEEN THE AMOUNT OF HETEROSIS, SHOWN BY THE PER CENT. INCREASE IN WEIGHT OF CROSSED SEEDS, AND THE SELECTIVE ACTION IN FAVOR OF THE PLANTS' OWN POLLEN. $r = +.496 \pm .093$.

Per Cent. Increase in Weight.

Deviation	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	41	43	
13.5					I				I												2
10.5	I																				1
7.5																					0
4.5																					0
−1.5																					0
+1.5	II		I							I				I		I					6
4.5							I				I										2
7.5	I		I		I																3
10.5																					0
13.5				I			I														2
16.5																					0
19.5										I											1
22.5																					0
25.5																					0
28.5																					0
31.5																					0
34.5																					0
37.5																					0
40.5																					0
43.5																					0
46.5									I			I								I	3
	4	0	2	1	2	0	2	0	2	2	1	1	0	1	0	1	0	0	0	1	20

POLLEN MIXTURE NO. 1.

A×A.	A×B.	B×A.	B×B.	C×A.	C×B.
317	1	319	5	361	10
189	2	224	9	429	15
270	7	369	10	421	13
389	8	348	26	445	1
332	8	330	6		
260	1				
1,757	27	1,590	56	1,656	39
1.21	7.69	0	22.22	.39	6.66
21	2	0	12	6	3
1,738	46	1,602	44	1,653	42
97.420	2.580	97.330	2.670	97.520	2.480
97.375	2.625	97.375	2.625	97.375	2.625
+.045	−.045	−.045	+.045	+.145	−.145

POLLEN MIXTURE NO. 2.

A×A.	A×B.	B×A.	B×B.
236	11	359	42
206	9	353	20
188	15	380	42
141	3	230	15
194	8	372	39
237	11	214	12
232	11	286	36
167	7	265	19
151	3	273	31
188	8	341	29
245	7		
2,185	93	3,073	285
2.69	8.00	0	2.34
59	7	0	7
2,133	145	3,080	278
93.630	6.370	91.720	8.280
92.675	7.325	92.675	7.325
+.955	−.955	−.955	+.955

The results, taken as they stand, are the same as obtained from maize. There is a deviation favoring the plants' own pollen of 2.06 and 6.84 per cent., in the two cases with the probability values .907 and .082 respectively. In the first instance the difference can easily be due to random sampling, in the other the odds are strongly against such an explanation. In view of the fact that differences in germination of the seeds and viability of plants most certainly tend to decrease the proportion of self-fertilized individuals, the data have some value. They should be corroborated by larger numbers using other characters which can be more surely identified before they are as convincing as the results with maize.

POLLEN MIXTURE No. 3.

A × A.	A × B.	B × A.	B × B.
23	3	90	41
54	7	114	28
27	0	27	26
34	1	15	20
18	0	97	56
43	2	73	19
19	0	76	52
4	0	27	8
7	1	50	3
		41	14
		66	55
		33	5
		22	11
		33	17
229	14	764	355
0	0	0	1.75
0	0	0	6
229	14	770	349
94.240	5.760	68.810	31.190
81.525	18.475	81.525	18.475
+12.715	−12.715	−12.715	+12.715

POLLEN MIXTURE No. 4.

A × A.	A × B.	B × A.	B × B.
189	38	301	67
173	42	208	102
165	11	248	70
64	14	164	167
41	7	194	38
77	15	254	117
		213	38
		278	49
709	127	1,860	648
0	.99	.83	1.72
0	1	15	11
710	126	1,856	652
84.930	15.070	74.000	26.000
79.465	20.535	79.465	20.535
+5.465	−5.465	−5.465	+5.465

POLLEN MIXTURE No. 5.

A × A.	A × B.	B × A.	B × B.
23	2	219	8
123	3	246	14
56	1	313	24
143	0	352	19
244	0	160	6
589	6	1,290	71
98.990	1.010	94.780	5.220
96.885	3.115	96.885	3.115
+2.105	−2.105	−2.105	+2.105

POLLEN MIXTURE No. 6.

A×A.	A×B.	B×A.	B×B.
40	71	184	129
—	—	0	2.63
—	—	0	3
40	71	187	126
36.04	63.96	59.74	40.26
47.89	52.11	47.89	52.11
−11.85	+11.85	+11.85	−11.85

POLLEN MIXTURE No. 7.

A × A.	A × B.	B × A.	B × B.
23	89	253	188
		104	189
		185	212
		84	117
		166	174
		115	139
		118	156
		123	190
		116	169
		109	158
		134	148
23	89	1,507	1,840
20.540	79.460	45.030	54.970
32.785	67.215	32.785	67.215
−12.245	+12.245	+12.245	−12.245

POLLEN MIXTURE No. 8.

A × A.	A × B.	B × A.	B × B.
96	53	173	108
158	112	98	69
154	60	74	83
136	47	102	102
106	38	175	131
103	29	199	147
135	65	109	79
89	53	196	89
130	47	142	153
60	11	203	131
128	49	117	93
63	38	52	52
69	29	166	106
87	34	109	79
186	67	83	72
59	31	138	86
103	54	133	138
77	34	76	75
124	51	45	41
58	18	119	116
64	36	75	108
2,185	956	2,584	2,058
0	0	0	1.69
0	0	0	35
2,185	956	2,619	2,023
69.56	30.44	56.42	43.58
62.99	37.01	62.99	37.01
+6.57	−6.57	−6.57	+6.57

Pollen Mixture No. 9.

A × A.	A × B.	B × A.	B × B.
277	149	209	115
116	69	173	99
168	69	264	156
135	78	188	114
151	52	160	101
78	30	210	142
128	71	172	134
254	102	191	160
286	147	166	112
208	97	195	134
185	88	196	107
113	55	175	108
208	117	207	105
277	130	128	97
		92	73
		123	76
		123	86
2,584	1,254	2,972	1,919
2.54	2.54	1.67	0
66	32	50	0
2,550	1,288	2,922	1,969
66.44	33.56	59.74	40.26
63.09	36.91	63.09	36.91
+3.35	−3.35	−3.35	+3.35

Pollen Mixture No. 10.

A × A.	A × B.	B × A.	B × B.
344	331	189	397
200	272	195	447
284	250	189	522
275	282	233	399
		169	430
1,103	1,135	975	2,195
1.71	0	1.69	1.71
19	0	16	38
1,084	1,154	997	2,173
48.440	51.560	31.450	68.550
39.945	60.055	39.945	60.055
+8.495	−8.495	−8.495	+8.495

Pollen Mixture No. 11.

A × A.	A × B.	B × A.	B × B.
67	57	209	130
385	203	290	199
		279	265
		386	191
		341	312
452	260	1,505	1,097
.88	0	0	0
4	0	0	0
448	264	1,505	1,097
62.92	37.08	57.84	42.16
60.38	39.62	60.38	39.62
+2.54	−2.54	−2.54	+2.54

POLLEN MIXTURE NO. 12.

A×A.	A×B.	B×A.	B×B.
552	196	218	106
86	67	197	92
265	111	163	69
253	114	105	46
182	61	66	56
401	155		
1,739	704	749	369
.86	0	0	0
15	0	0	0
1,724	719	749	369
70.57	29.43	66.99	33.01
68.78	31.22	68.78	31.22
+1.79	−1.79	−1.79	+1.79

POLLEN MIXTURE NO. 13.

A×A.	A×B.	B×A.	B×B.
37	52	14	11
17	87	13	18
11	16	17	11
28	86	6	4
50	91	31	8
42	59	14	13
185	391	95	65
32.120	67.880	59.370	40.630
45.745	54.255	45.745	54.255
−13.625	+13.625	+13.625	−13.625

POLLEN MIXTURE NO. 14.

A×A.	A × B.	B × A.	B × B.
41	13	58	31
91	35	35	15
83	39	46	10
70	27	17	6
139	36		
424	150	156	62
73.870	26.130	71.560	28.440
72.715	27.285	72.715	27.285
+1.155	−1.155	−1.155	+1.155

POLLEN MIXTURE NO. 15.

A×A.	A×B.	B×A.	B×B.
309	11	3	111
415	11	0	53
393	9	1	71
326	7	1	113
276	2	0	67
135	1	2	90
400	4		
355	2		
2,609	47	(7)	(505)
		14	498
98.23	1.77	2.73	97.27
50.48	49.52	50.48	49.52
+47.75	−47.75	−47.75	+47.75

Pollen Mixture No. 16.

A×A.	A×B.	B×A.	B×B.
285	5	12	355
187	1	8	350
251	2	8	408
		4	226
		5	117
723	8	(37)	(1,456)
		74	1,419
98.910	1.090	4.960	95.040
51.935	48.065	51.935	48.065
+46.975	−46.975	−46.975	+46.975

Pollen Mixture No. 17.

A × A.	A × B.	B × A.	B × B.
255	1	4	49
166	0	0	51
185	1		
222	0		
220	1		
255	0		
1,303	3	4	100
99.77	.23	3.85	96.15
51.81	48.19	51.81	48.19
+47.96	−47.96	−47.96	+47.96

Pollen Mixture No. 18.

A × A.	A × B.	B × A.	B × B.
3	5	1	368
1	16	0	432
		0	320
		0	336
		0	39
		0	78
4	21	1	1,573
16.00	84.00	0.06	99.94
8.03	91.97	8.03	91.97
+7.97	−7.97	−7.97	+7.97

Pollen Mixture No. 19.

A × A.	A × B.	B × A.	B × B.	C × A	C × B.
20	16	41	206	75	213
89	53	36	147	71	124
68	86	42	200	61	315
55	47	45	178		
69	39	29	150		
45	20	63	156		
52	37	30	88		
65	8	57	218		
65	86				
528	392	343	1,343	207	652
57.390	42.610	20.340	79.660	24.100	75.900
38.865	61.135	38.865	61.135	38.865	61.135
+18.525	−18.525	−18.525	+18.525	−14.765	+14.765

POLLEN MIXTURE NO. 20.

A × A.	A × B.	B × A.	B × B.	C × A.	C × B.
172	11	193	86	40	52
43	6	104	58	46	56
165	15	117	86	51	55
38	2	80	45	2	5
89	4	47	17		
103	8	128	52		
82	12	81	47		
19	0	94	60		
53	4	92	30		
33	0	99	43		
69	13	75	46		
31	2	64	35		
897	77	1,174	605	139	168
92.09	7.91	65.99	34.01	45.28	54.72
79.04	20.96	79.04	20.96	79.04	20.96
+13.05	−13.05	−13.05	+13.05	−33.76	+33.76

POLLEN MIXTURE NO. 21.

A × A.	A × B.	B × A.	B × B.
39	16	17	25
12	14	1	5
32	25	36	18
14	18		
97	73	54	48
57.06	42.94	52.94	47.06
55.00	45.00	55.00	45.00
+2.06	−2.06	−2.06	+2.06

POLLEN MIXTURE NO. 22.

A × A.	A × B.	B × A.	B × B.
7	22	13	17
10	27	7	27
107	103		
124	152	20	44
44.93	55.07	31.25	68.75
38.09	61.91	38.09	61.91
+6.84	−6.84	−6.84	+6.84

PREVIOUS INVESTIGATIONS ON SELECTIVE FERTILIZATION.

From the work of Kölreuter, Herbert, Gärtner, Darwin, Müller, Knuth and others we are familiar with the phenomenon of self-sterility in plants in which the individual's own pollen is wholly incapable of functioning on the plant by which it is produced although perfectly developed and able to fulfill its duties when brought to other plants of the same species. Numerous investigators have been giving attention to this problem in recent times. East and Park (1917) have made a noteworthy contribu-

tion to its solution and give a complete résumé of the work which has been done on this subject. They have been able to demonstrate that groups exist within which the individuals are all both self-sterile and cross-sterile, but any member of one group is perfectly fertile with any member of any other group. These investigators find that there are about 100 well-endorsed instances of self-sterility in plants scattered over some 35 families. Undoubtedly this discrimination is a means to promote cross-fertilization of approximately the same significance as floral contrivances, dichogamy and diœcism. Even though widespread in its occurrence self-sterility is a special adaptive process fulfilling a particular function. It is directly opposite in its effect to the results found in maize which shows no self-sterility of the type found in Nicotiana and other genera. At least no clear cases are known of maize pollen, which is unable to fertilize the plants which produced it, being able to fertilize other plants.

Darwin has furnished many instances of self-sterility. In addition he reports some experiments which led him to believe that even when a plant was normally self-fertile that pollen from unrelated plants of the same species was prepotent over the plant's own pollen. In discussing means which insure flowers being fertilized with pollen from distinct plants, he says: "We now come to a far more general and therefore more important means by which the mutual fertilization of distinct plants is effected, namely, the fertilizing power of pollen from another variety or individual being greater than that of a plant's own pollen. The simplest and best known case of prepotent action in pollen, though it does not bear directly on our present subject, is that of a plant's own pollen over that from a distinct species. If pollen from a distinct species be placed on the stigma of a castrated flower, and then after the interval of several hours, pollen from the same species be placed on the stigma, the effects of the former are wholly obliterated, excepting in some rare cases. If two varieties are treated in the same manner, the result is analogous, though of directly opposite nature; for pollen from any other variety is often or generally prepotent over that from the same flower" ("Cross- and Self-Fertilization," pp. 391–392).

These statements were based on observations and experiments

with various cultivated plants. Different types of crucifers—kohl-rabi, borccoli, Brussels sprouts, cabbage—were grown near each other and the seed resulting from pollination at will, when grown, showed a large amount of intercrossing. The observation was also made with different varieties of the radish, *Raphanus sativus*. These plants are all partially self-sterile so that cross-fertilization is expected in somewhat greater degree than would result from random pollination. Mixing was also shown by plants which are generally self-fertile such as tulip, hyacinth, anemone, ranunculus, strawberry, orange, rhododendron, rubbarb. The fact that vicinism occurs when varieties of these plants are grown together is established by such observations but this does not prove that one type of pollen is prepotent over the other. Somewhat more significant results were obtained from two other species. *Mimulus luteus* was found to be highly fertile when insects were excluded. Uncastrated flowers of a constant whitish variety were artificially pollinated by a yellowish variety and of the 28 resulting plants all had yellowish flowers so that the "pollen of the yellow variety completely overwhelmed that from the mother plant." A crimson variety of *Iberis umbellata*, which was self-fertile, was crossed with a pink variety, the pollen being applied to uncastrated flowers as before upon the stigmas of which he saw abundant pollen presumably from the same flowers. Out of 30 plants raised 24 showed themselves to be crossed by the altered color of their flowers.

Obviously experiments such as these are not sufficient to establish the prepotency of foreign pollen in self-fertile plants. A number of conclusions might be drawn from such results. The cross-fertilized seeds may have germinated better and the plants grown from them survived in greater numbers. The types may not have been as constant for their flower color as Darwin supposed or the ovules may not have been receptive at the time the plant's own pollen was available but were when the foreign pollen was applied. Taken as they stand the results do indicate a prepotency of pollen from dissimilar plants and it would be desirable to investigate this effect with these species using mixed pollen in reciprocal applications as employed with maize and the tomato.

Darwin knew of many cases of total self-sterility and was so convinced of the necessity for cross-fertilization that he was easily persuaded from these observations that a prepotency of pollen from unrelated plants did exist since he supposed this enabled a plant to choose between its own and unrelated pollen when both were brought at the same time to the stigmas by insects or other agencies. So plausible have been the arguments in favor of such an assumption that the prepotency of germ cells from individuals of somewhat different constitution, even where complete self-fertility exists, has been accepted as an established fact and incorporated in textbooks on biology.

Similarly inconclusive experiments have been performed with animals. Marshall (1910) artificially impregnated a pure bred dog with a mixture of equal quantities of seminal fluids from the same breed and from a mongrel of unknown ancestry. Of the four young which resulted one died early, and three resembled somewhat the mongrel sire. Marshall cites another instance in which a dog copulated with a member of the same breed and two days later with a sire of different type. Out of three puppies one was pure bred and two half-breeds. These cases, according to this writer, indicated a selective action favoring dissimilar rather than related spermatozoa. King (1918) mentions some preliminary experiments with albino and wild gray rats in which advantage was given to the former, yet the results tended to show a prepotency of the latter, so that there was apparently a selective action favoring the out-cross. The details of these experiments are not given.

In attempting to determine whether or not a selective action exists small numbers can never be more than suggestive and unless the mixture is applied at the same time to both types furnishing the sperm cells there is no way of estimating the relative proportions of the two kinds of fertilizing elements present in the mixture which are capable of functioning. Furthermore a constant excess of cross-fertilized individuals over the others may be due to the greater viability of the hybrids and hence there will be a selective elimination of zygotes but not necessarily slective fertilization. Hyde (1914) compared the matings of different types of Drosophila within the strains and in reciprocal

crosses. The dissimilar unions gave greatly increased numbers in both reciprocal combinations. However, the type of mating did not influence the number of eggs laid and there is no proof that cross-fertilization occurred more readily than self-fertilization. The results show that the cross-fertilized eggs hatched better and the offspring survived in greater numbers, a result which is easily understood since there were lethal factors involved in the material worked with.

The only evidence from the animal side of a definite selective action comparable to the many instances of self-sterility in plants is the well-known case of self-impotency in *Ciona intestinalis* (Castle, 1896). Morgan (1905, '07, '10) has experimented with this organism and has found that the self-sterility is not always complete. Material gathered on the Pacific coast showed somewhat greater receptiveness to the individual's own sperm than eggs of the same species at Woods Hole which were almost entirely unresponsive to sperm from the same individual which produced the eggs. In another ascidian, *Cynthia partida*, he found that self-fertilization takes place frequently but the sperm of unrelated individuals is more effective. A third species, *Molgula manhattensis* is self-fertilized as readily as cross-fertilized. From this it seems that incompatibility of uniting gametes as a means of insuring cross-fertilization exists in various grades of effectiveness. Even in extreme cases the degree of self-sterility may be modified by internal and external conditions. In Nicotiana East and Park find that self-fertilization sometimes takes place towards the end of the growing period when the vigor of the plants is reduced.

Discussion.

As far as the writer knows the results obtained from maize stand alone among plants in showing a selective action unfavorable to fertilization by sperm from individuals of different hereditary constitution. The handicap placed upon the foreign pollen is proportional to the germinal unlikeness. If the unequal effect is due to a slower growth of the pollen tube through the tissues of style the selective action may be restricted to plants,[1]

[1] E. C. Miller (Jour. Agric. Research, Vol. 18, pp. 255–266, Dec. 1919), has recently made a detailed study of fertilization in maize and finds that from many

and would also not be surprising to find that the phenomenon is greater in maize than in any other species for the reason that in this plant the pollen tubes have a larger distance to traverse to accomplish fertilization than in any other form known to the writer.

The stigmatic hairs of maize are scattered along a filamentous style which continues to grow until fertilization takes place. The structure withers and dries shortly after pollination takes place. Pollen may adhere at any point along the filament. The total length of style through which the pollen tube grows is normally from 10 to 20 centimeters but in extreme cases may be as great as 50 or more. It will be worth while to see whether or not the selective effect is more pronounced when the styles are long than when short. Such an experiment can be easily carried out and would give some indications as to whether the handicap is placed during pollen tube growth or after the sperm nuclei are brought to the egg.

The lessened ability of moderately different types to fertilize is in line with the impossibility of effecting unions between widely separated forms. In such cases the prevention of fertilization is sometimes due to mechanical difficulties in the way of bringing sperm cells to the eggs but even when this is accomplished there still exists a firm barrier which prevents the passage through the egg membrane. The differential effect demonstrated in maize may be simply a reduced manifestation of this phenomenon.

It is possible that the experiments on anaphyllaxis may throw some light on this problem. It is known that foreign proteins when injected in animal tissues may have a toxic effect and excite an extreme irritability so that in repeated doses they may cause markedly injurious results. By this means it is possible to distinguish between proteins of very slight differences in composition. Since the differences in protoplasmic substances between the types in which a selective action is shown seem to be small there may be some relation between the two phenomena. However, the male gametophyte growing upon the stigmas is, in

pollen tubes which start to grow down the style only one tube in every case in nearly 100 observations was seen to reach the ovary cavity. This indicates that the differential fertilizing power is determined by the rate of pollen tube growth and not after the sperm nuclei have been brought to the egg.

a way, merely a parasitic organism. It would be difficult to find evidence from true parasites that they are restrained by their hosts in proportion as they are genetically dissimilar although in most cases the differences are so great that probably there is no basis for comparison.

The only evidence which has any direct bearing on the problem comes from grafting experiments. It is well known with plants that the affinity of stock and cion is directly proportional to their phylogenetic similarity. In animals the same rule holds, and very fine distinctions are manifested. Morgan (1910) cites the results of Schoene in which the skin of the mouse is readily grafted back upon the same individual or member of the same litter but not upon unrelated mice. Such results as these are quite similar to the greater receptiveness of plants to their own pollen.

For some time there has been current in biological literature the hypothesis that heterogeneity in the structure of organisms favors growth and reproduction and conversely that homogeneity is unfavorable. This is a heritage from Darwinism and has appeared again and again in theories of rejuvenation, vigor derived from crossing, and selective fertilization. It has been stated in many different ways but in general terms it amounts to the supposition that similarity in protoplasmic structure brought about by a line of similar ancestors is not conducive to physiological efficiency and that the differences brought about by the union of unlike elements and the consequent lack of balance stimulates growth. The only basis for this hypothesis is found in the necessity, in most cases, for the union of two differentiated sex cells to start the development in the egg. The attempt to argue by analogy than the union of dissimilar protoplasms is, in itself, an immediate physiological benefit is not supported by the facts and is founded upon fallacious reasoning.

Rejuvenation in vegetatively reproduced organisms by sexual union is no longer looked upon as beneficial in destroying similarity in structure. That the process of forming gametes and their reunion may bring about a reorganization of the protoplasmic substances and an elimination of waste products so as to result in greater growth seems quite plausible. But the

significance of such a process is not to be looked for in the bringing together of differentiated germplasms.

The advantage derived from cross-fertilization is now understood as a phenomenon of inheritance and the older hypothesis of the stimulation of heterozygosis is no longer needed. According to present theory homozygous factor combinations are more efficient than heterozygous combinations of the same factors. In the lowest organisms which are illustrative of a primitive sexuality there is direct proof that the union of different individuals does not result in an increased developmental efficiency. Jennings (1913) finds that in Paramecium, in the generations immediately following conjugation, there is a slowing down in the rate of division. The advantage derived from the pairing of individuals is the greater elasticity in adaptiveness resulting from the mixing of different germ plasms giving to some of the descendants a greater chance for survival. Pearl (1907) has found that in this same organism there is a tendency for like forms to conjugate due to mechanical hindrances to the pairing of individuals dissimilar in form. This has been substantiated by Jennings (1911). Also in gastropods Crozier (1918) has demonstrated that assortative mating takes place between individuals of the same size and this, he considers, results in a greater number of offspring than there would be if random pairing was the rule. It has been proven by Pearson and Lee (1903) that assortative mating occurs in man. This conclusion is reached after extensive investigation in the inheritance of physical characters in which they have found that there are positive correlations between husband and wife with respect to stature, span of arms, and length of forearm. Moreover they have shown that homogamy is a factor favorable to fertility. Parents with like characters are more productive of offspring. This is an important observation and supports the main thesis of this paper.

The occurrence of homogamy in such widely diverse forms of life as the higher plants, protozoa, mollusca and man cannot fail to have significance. The importance of discriminate isolation has long been recognized since it supplies a necessary factor in divergent evolution. The existence of such an assortative action can now be looked for in all forms of life since evidence has been

produced from the corners of the phylogenetical triangle. The evolutionary significance of this phenomenon has been ably reviewed by Peal in the above citation so that it is not useful to go into that phase of the problem in detail here. It is interesting to note that the results from maize fulfill his expectation as in conclusion he says, "the fact that we find such a high degree of homogamy in a protozoan form like Paramecium strongly suggests the possibility that in higher organisms there may be assortative mating of the gametes in the process of fertilization. Should such a homogamy occur it would probably be of far greater importance than any assorted mating of the somas." While the selective agency in plants does not differentiate between gametes produced by one type in the end the result is the same. Individuals with like characters tend to be brought together and virtually to be set apart from the general population.

Biological investigations unite to show that the importance of sex is to make organisms more plastic in adaptability. The advantages have been so great that sexual reproduction is now established as the dominant method for the renewal of organism in both kingdoms at the expense of economy and speed of multiplication. It is not strange then that accessory devices have been developed to insure the fulfillment of the function for which so much has been expended. Self-sterility or self-impotency is one of the means developed to serve this purpose.

The reverse phenomenon, that of self-prepotency, so far is shown among plants only by maize. It would be surprising to find it limited to this one species. Is it not more likely to be a general manifestation? As a fundamental principle it may apply even to those organisms which show self-sterility, this latter being a special adaptation entirely overcoming the handicap placed upon unfamiliar gametes in order to make certain the advantages which exogamy holds out. One cannot insist upon such an assumption with evidence from only one or possibly two species. But the evidence, limited though it may be for the present, is one more indication that homogeneity, similarity, likeness, familiarity, or however it may be described, in protoplasmic structure is consistent with and favorable to the highest physiological efficiency.

In maintaining two opposing tendencies Nature is not necessarily working at cross-purposes. Biparental inheritance with the inclination towards exogamy serves to bring about plasticity. The preferential mating of similar kind operates to make this mixing discriminative. Probably it is not yet time to reconcile completely these two contradictory forces. It may at least be held that assortative mating which favors the pairing of like with like has some importance in evolution since it is an agency in orthogenetic changes. Perceptive reproduction of this kind tends to hold organisms in certain paths once a break from the common type has been made. Having been demonstrated in three of the farthest separated branches of the organic world—angiosperms, protozoa, and man—homogamy may take a somewhat more authoritative part in evolutionary theory.

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STUDIES ON THE CELLS OF CATTLE WITH SPECIAL REFERENCE TO SPERMATOGENESIS, OÖGONIA, AND SEX-DETERMINATION.

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I. INTRODUCTION.

Sex-determination with its many attendant problems has always been a subject of great interest to practical animal breeders; and the art of breeding has always been replete with rules by which the sex ratio might be shifted in various ways to the advantage of the breeder. These rules, however, have been founded upon inadequate evidence and unsound reasoning.

The most common beliefs in regard to sex control have from time to time been founded on heat relations, some maintaining that the products of conception in early heat were more often males, others that they were more often females. Pearl and Parshley ('13) have published data on sex-determination in cattle with the following conclusions: (1) That as the time of coitus approaches the end of the œstrous period there is a pro-

[1] This research was supported in part by the Adams Fund of the Idaho Experiment Station.

gressive increase in the proportion of male young born. (2) That in the extreme case this increase in the proportion of male births is probably statistically significant and not to be attributed to errors of random sampling. (3) That these modifications of the sex ratio cannot be attributed to age differences or to any other factor yet suggested.

More recent and extensive data, however, according to Pearl ('17) make the relation of time of service to sex extremely doubtful. He says: "The apparent relation between these two factors, which is believed by many breeders to exist and which our earlier statistics appeared to indicate, seems now to be purely accidental, and to have arisen only because of the comparative meagerness of the statistics on which the matter was discussed." Sex in cattle is a matter of heredity, as is shown by the results of this investigation, and remains a matter beyond the control of the breeder; its ultimate control is problematical. However, the suggestion of experimentally separating the two types of spermatozoa, or destroying the one type without impairing the nature of the other, does not appear to be entirely hopeless. If this can be done, the problem of sex control will be comparatively simple, since artificial insemination can be resorted to.

It appears to be a well-established fact that sex is determined at the time of fertilization. Sex, like other characters of the individual, has a definite factorial basis; and the factorial constitution of the individual with respect to sex as well as to other characters is fixed by the constitution of the two gametes which unite to form the zygote.

The dimorphic condition among the spermatozoa in many of the lower animal forms is well known. Guyer pointed it out in the rat ('10), and man ('10); other investigators have reported it in several other mammals. Among the domestic mammals the same condition has been clearly demonstrated in the pig (Wodsedalek, '13) and in the horse (Wodsedalek, '14). This study on the sex cells of cattle shows that two types of spermatozoa are also produced in the bull; the one type at the time of fertilization determining maleness and the other type determining femaleness. The spermatozoan which determines femaleness is somewhat

larger owing to the presence of a large sex-chromosome or accessory element which is lacking in the type producing maleness. Since it has been found in this study that the oögonia of cattle possess two sex-chromosomes it is safe to conclude, in view of our knowledge of oögenesis in general, that all of the mature ova carry the reduced number or one sex-chromosome. If the ovum is fertilized by a sperm which lacks the sex-chromosome the resulting zygote naturally possess only one sex-chromosome and, therefore, develops into a male. This is evidenced by the fact that the somatic as well as the germ cells of the male possess a single accessory element. On the other hand, if the ovum is fertilized by a sperm possessing the sex-chromosome the resulting zygote possesses two sex-chromosomes and, therefore, develops into a female. The somatic as well as the germ cells of the female possess the two accessory elements which is in exact accord with expectations.

The results presented in this paper were obtained through a most careful and critical study extending over a period of more than four years. The problem was started in the spring of 1915 and every summer since and a great deal of the spare time in the intervening school years, with the exception of the year 1918, has been devoted to it. In the aggregate this means a total of about fifteen months of continuous work. Practically all of the results presented in this paper were at hand at the end of the first two and a half years of study. Over four hundred pencil sketches were made of the various cells, especially those in mitosis, with the aid of the camera lucida and the location of each, as indicated by the mechanical stage, was carefully recorded in a separate booklet. A brief summary of the results of the study was written up and all of the material was put away for a whole year (1918) during which the summer months and other spare time was devoted to the study of the sex cells in another mammal. No effort was made to remember any of the results of the study on the sex cells in cattle.

In the meantime, considerable new cattle material was obtained and hundreds of new slides were made by the departmental technician. At the end of the year the new cattle material was studied and the results were carefully recorded. The original

material was then reinvestigated and new sketches were made of many of the cells, the location of which was recorded before. The results obtained through the study of new slides prepared by the technician, as well as those obtained through the second study of my original material were then compared with the original results. Several hundred different germinal and somatic cells from both sexes were carefully checked up. Especial attention was paid to the number of the ordinary chromosomes and to the sex-chromosomes. Only in a comparatively few cases was there any discrepancy in the interpretation of the nature and number of the ordinary chromosomes. Conditions pertaining to the sex-chromosomes were corroborated in every instance. I was further checked up on my chromosome counts by several of my assistants and senior pre-medical students who had considerable training in microscopic anatomy. In general, these men who knew nothing of my own interpretations, corroborated my counts in a surprisingly large number of instances.

II. Material and Methods.

All of the material used in this investigation with the exception of some ovaries was obtained through the courtesy of the management of the Hagan and Cushing Packing Plant which adjoins the University farm. Some excellent ovarian tissue was given to me by my colleague Dr. A. R. Hahner, formerly professor of veterinary science of the University of Idaho. The ovaries were removed from two five-months-old heifers of the university herd. Testes were obtained from seven mature bulls and one male fœtus of five months and from six smaller fœtuses varying from two to eight and one half inches in length. The ovaries were obtained from four heifers and as many cows, and from five small fœtuses, varying from two and one half to seven inches in length. In addition to this six small embryos were sectioned. Many slides were also made of various somatic structures from the small fœtuses of both sexes.

Several fixing fluids were tried on the testicular material, including Hermann's, Gilson's, Flemming's, and Bouin's. Bouin's fluid used straight or with slight modifications, at 38° C. was the most universally successful fixing agent. When used at some-

what lower temperatures, the material showed little or no modifications. When Bouin's fluid was modified, the alteration took the form of the addition of a small amount of chromic acid, or urea, or the reduction of the percentage of acetic acid. All of these slight modifications gave very good results in the testicular material, and the ovarian, embryological and fœtal tissues appeared to be best when fixed in the fluid modified with chromic acid. The cold method (Hance '17) was also tried in two instances with the testicular tissue but with less success. And while I have not tried this out on the cattle tissue myself, I have every assurance that the laboratory technician carried out the process with great care.

In the study of the male germ cells, smears as well as sections were used. Many stains and counter stains were tried. Iron-hæmatoxylin when used alone was found to be the most satisfactory. All of the figures represented in this paper were made from material stained in this manner.

III. General Arrangement of the Male Germinal Cells.

The structure of the testes of the bull is similar to that of the other well-known mammals and bears a great resemblance to the conditions found in the testes of the horse (Wodsedalek '14). The interstitial cells as in the horse are small and fewer in number in comparison with their large size and great abundance in the testes of the pig. The size of the seminiferous tubules, as well as the general size of the various germinal cells, however, corresponds to the condition found in the pig. The usual types of cells, (1) spermatogonia, (2) primary spermatocytes, (3) secondary spermatocytes, (4) spermatids, and (5) spermatozoa in various stages of development, are present in great abundance.

IV. Spermatogenesis.

In general the spermatogenesis of the bull corresponds to that of the pig and the horse. Since many of the finer cytological points are given in detail in the papers on the pig and the horse (Wodsedalek '13 and '14) they are omitted here to avoid unnecessary duplication. And while all of the finer details involved in a thorough piece of work in spermatogenesis were carefully

studied in this animal, only the phases pretaining to the chromosome numbers and their behavior are emphasized in this paper.

1. *Spermatogonia.*

The spermatogonia usually lie in a single layer next to the wall of the tubule, though occasionally some of the cells are crowded out, thus forming a second layer which is always very irregular. The cells which undergo the last spermatogonial division (Figs. 5–12) are usually beyond the first layer, though occasionally they may be found next to the tubule wall along the entire section of the tubule. At times the cells are far apart, in which case they are flattened out on the tubule wall. The cells also differ considerably in size and appearance, depending on the stage of development they are in.

During the resting stage a large nucleolus is invariably present. As a rule it assumes a somewhat heart-shaped appearance; especially is this true in the larger cells and in those in which the chromosomes begin to form. At the conclusion of the resting stage numerous large chromatin granules appear and arrange themselves along fine threads in an entangled mass. The chromosomes soon become distinct and mitotic figures are fairly numerous. And while, as a rule, there is considerable overlapping and massing of the chromosomes in the early spermatogonial divisions, hundreds of cells were found in which there was little or no overlapping, making accurate counts possible.

Thirty-seven chromosomes appear in the late prophase of the spermatogonial division (Fig. 1). Thirty-six of these are variously shaped, mainly oblong or slightly curved, and differ somewhat in size. One which is much larger is triangular in form or heart-shaped. This is the accessory or sex-chromosome, and is the same thing as the large nucleolus which appears in the resting stages. This point is certain, as the body can be easily traced through the various stages of the cells. A similar condition was reported by Guyer ('10) in man, Wodsedalek in the pig ('13) and in the horse ('14). Several other investigators have reported it in other forms since. During division each chromosome, including the sex-chromosome, divides in two (Figs. 3 and 4).

In the last spermatogonial cells the chromosomes appear in a dense mass. The cells gradually increase in size and great expansion takes place in the nuclei (Figs. 5–9). The chromosomes become decidedly distinct and surprisingly well segregated throughout the spherical nucleus (Figs. 10 and 11). The cells in these stages are numerous and beautiful. The chromosomes appear to be dense in structure and are thicker than those of the early spermatogonial cells. Several of the chromosomes are almost spherical. They are so evenly distributed that hundreds of accurate counts can be made within a short time. This condition prevailed in all of the mature testes studied and the cells were especially numerous in three two-year-old bulls. Even in somewhat stale tissue (Fig. 12) the chromosomes in these cells appear to remain well segregated. Altogether over one thousand accurate counts were made in these cells alone.

2. *Primary Spermatocytes.*

The sex-chromosome can invariably be seen in the spermatocytes where it retains its individuality (Fig. 13). Just how pairing takes place in these cells cannot be stated with certainty. When the chromosomes appear for division they are of the reduced number and bivalent in nature. The thirty-six ordinary chromosomes pair while the sex-chromosome remains unpaired and can easily be distinguished from the others (Figs. 14–18). It occasionally shows its double nature in the late prophase (Fig. 17), and more frequently in the later stages of the primary spermatocyte division (Figs. 22, 24, 25, 27, 28 and 29).

During the primary spermatocyte division the sex-chromosome usually passes to one pole in advance of the other chromosomes (Figs. 19–25). This unequal division of the chromosomes in these cells (Figs. 19–29) gives rise to two different types of secondary spermatocytes. The one type containing the eighteen ordinary chromosomes plus the sex-chromosome. Just before division is complete the chromosomes become loosely paired (Figs. 28 and 29). This peculiar behavior of the chromosomes is apparently quite common in mammalian tissue.

3. *Secondary Spermatocytes.*

No resting stage occurs in the secondary spermatocyte. This condition also occurs in the horse and is frequently found in the pig, according to my former studies. The secondary spermatocytes divide soon after they are formed and not infrequently the spindles are formed in the two cells resulting from the first spermatocyte division while they are still in close contact. Nine chromosomes arrange themselves in the equatorial plate for division in the one type of secondary spermatocyte (Fig. 31), and nine plus the sex-chromosome in the other (Fig. 30). All of the chromosomes, including the sex-element when it is present, divide in these cells (Figs. 32–38).

4. *Spermatids.*

The division of the secondary spermatocyte gives rise in the one case to spermatids containing nine chromosomes (Figs. 36–38), and in the other case nine plus the sex-chromosome or ten (Figs. 33 and 34). All of the chromosomes except the sex-chromosome are bivalent in nature so that in reality we have the equivalent of eighteen chromosomes in the one kind of spermatid and eighteen plus the sex-chromosome in the other. The bivalent chromosomes frequently begin to separate before the division of the cell is complete. Occasionally the eighteen chromosomes can be distinguished as independent elements after the cell divides (Fig. 38), although the chromosomes usually disintegrate before complete separation can be identified. All of the foregoing evidence indicates that eighteen is the reduced number of chromosomes.

V. Dimorphism in the Spermatozoa.

The spermatozoa of the bull vary considerably in size, and careful measurements show that they may be arranged in two separate classes, one type being much larger than the other. Mature specimens, which were free in the lumen of the tubule and parallel to the objective, were selected at random from a single slide and outline sketches of six hundred heads were made with the aid of a camera lucida. The lengths of the sketches were then carefully measured and recorded in quarter millimeters. Figure 1 in the text shows the variation in size of the six hundred

heads measured. It shows a distinct bimodal curve with modes at 12.50 mm., and 14.75 mm. The intermodal depression is

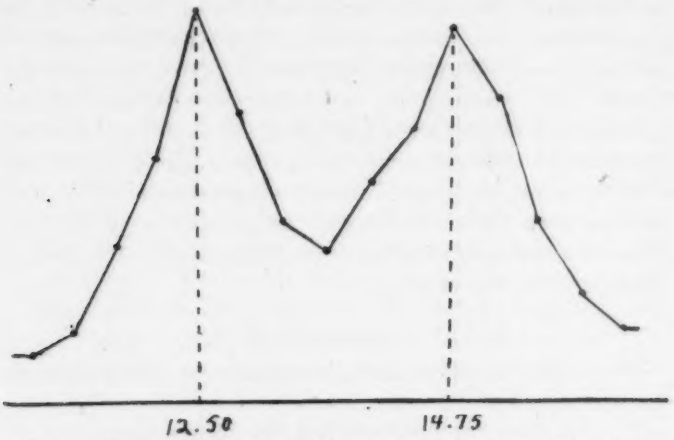

FIG. 1. Diagram showing the variation in size among six hundred mature spermatozoa of the bull.

deep and wide and the two elements of the curve are approximately equal as regards number of individuals. The spermatozoa of the larger type undoubtedly possess the sex-chromosome, while those of the smaller type are without it. Figures 39 and 40 show the comparative size of the two distinct types of mature spermatozoa. They can be distinguished with ease under a high-power microscope.

The general scheme of the development of the spermatozoön from the spermatid of the bull is similar to that in the pig and the horse (Wodsedalek '13 and '14) and, therefore, will not be described here. Bimodal curves were also shown by the writer in connection with the spermatozoa of the pig and the horse, and in a number of other species by Zeleny and Faust ('14 and '15) and by Zeleny and Senay ('15).

VI. OÖGONIA.

The best ovarian material studied in connection with the number of chromosomes in the oögonia was obtained from some of the small fœtuses. The results obtained from this material

were corroborated in the tissue obtained from cows and heifers, although the adult tissue was not nearly as satisfactory. Most excellent material was found in the ovaries of a four and a half inch fœtus and one six-inch fœtus. These were fixed in the Bouin's plus chromic acid fixing fluid. The oögonia were apparently in extreme activity at the time of fixation. Mitotic figures are abundant and the chromosomes are very distinct, especially in the late prophase and early metaphase stages which are numerous (Figs. 44–46). In general these cells resemble the last spermatogonial cells (compare Figs. 44 and 10), except that the oögonia are somewhat larger.

In well fixed and favorably stained material the oögonia in the resting stage invariably show two large nucleoli each corresponding to the single nucleolus of the resting stage of spermatogonial cells (Fig. 41). These retain their individuality during the spireme stage though at times they are somewhat distended (Fig. 42). When the spireme breaks up the chromosomes are long and narrow and variously curved (Fig. 43). In the late prophase they become shorter and thicker and appear evenly distributed throughout the large nuclei (Figs. 44 and 45). In this stage as well as in the early metaphase stages of division (Fig. 46) hundreds of accurate counts were possible. The two sex-chromosomes can be easily distinguished in all of the prophase stages (Figs. 41–45).

Thirty-eight chromosomes are present in the oögonia (Figs. 43–46). Thirty-six are the ordinary chromosomes corresponding to the thirty-six ordinary chromosomes in the spermatogonia of the male. The two other elements are the sex-chromosomes. When the chromosomes arrange themselves in the equatorial plate for division the sex-chromosomes are always at the periphery (Figs. 46 and 47). During division all of the chromosomes, including the sex-elements, divide in two (Fig. 48). All of the figures of the oögonial cells were made from the same section (Figs. 41–48).

On account of the great significance of the two sex-chromosomes in the femal tissue a tremenduous amount of time was devoted to this particular phase of the problem; this was also true of the studies in connection with the chromosomes of the

somatic tissue in both sexes. The final preparation of the paper for publication was postponed on several occasions, not at all because of any uncertainties, since the results were convincing from the start, but rather because each additional survey of the entire problem from new material proved more fascinating and gratifying than those preceding.

VII. Chromosomes in Somatic Cells.

Numerous slides were made of various somatic structures from fœtuses of both sexes. The organs most frequently used were the brain, lung, liver, Wolffian body, kidney, and intestine. The larger embryos were cut into pieces all of which were then sectioned. Three small embryos, ranging from ten to fourteen millimeter neck-lengths were sectioned in toto. While splendid mitotic figures and late prophase stages were found in many parts of the embryos the very best cells were found in the brain. In the larger specimens the liver and kidneys showed the most favorable cells.

The male somatic cells, like the spermatogonia, contain thirty-seven chromosomes, of which thirty-six are the ordinary chromosomes and one is the accessory element or sex-chromosome (Figs. 51 and 52). The female somatic cells, like the oögonia, contain thirty-eight chromosomes, of which thirty-six are the ordinary chromosomes and the other two are the sex-elements (Figs. 49 and 50). The sex-chromosomes in each case were as distinguishable as they are in the germinal cells. Literally thousands of somatic cells were carefully studied in each sex.

After the chromosomes of the first one hundred of the most favorable male somatic cells were studied and carefully sketched it was found that in ninety-three cases there were thirty-six of the ordinary chromosomes present plus the one accessory. In the other seven cases there were slight discrepancies, usually one or two less. This was in all probability due to unnoticeable overlapping. In two cases there were two extra chromosomes present. This was in all probability due to the fact that two of the chromosomes had divided, since these cells were in the early metaphase stage. This interpretation appears to be correct, since it was found later that occasionally some of the chromo-

somes divide considerably in advance of the others. This point was observed in several of the early metaphase stages; it was fairly common in polar views of late metaphase stages, and in a few instances a chromosome was found almost completely split in two even in the late prophase. For this reason, in selecting cells for accurate chromosome counts great care must be exercised not to select polar views of late metaphase stages. The increase in number may also be due to fragmentation, although one would expect this only in poorly fixed material or general poor technique.

After the counts in the first one hundred camera-lucida sketches showed that over ninety per cent. of the cells contained thirty-seven chromosomes, no more sketches were made. However, hundreds of other counts were made from favorable cells off-hand with about the same results. This shows that in at least ninety per cent. of the male somatic cells thirty-six ordinary chromosomes, and a single sex-chromosome were present. The ten per cent. of discrepancies is undoubtedly due to unnatural states and probable sources of error. The studies of the chromosomes in the somatic cells of the female were conducted in the same manner. Among the first one hundred cells, ninety-one showed thirty-six ordinary chromosomes and two accessories. In several hundred further counts, the percentage of discrepancies was about the same.

In the case of the six small embryos in which sex could not be determined morphologically, or was uncertain, one was poorly fixed, although it was cut into small pieces, and, therefore, could not be used in this study. Of the other five two were unquestionably female and three were male, according to evidences from the cytological standpoint. In the case of the two specimens, cells in various parts of the body repeatedly showed thirty-six ordinary chromosomes and two accessories. In many instances where the ordinary chromosomes could not be counted, the two sex chromosomes were recognizable. In the other three specimens the cells invariably showed only a single sex-chromosome, and in many cases the thirty-six ordinary chromosomes were counted.

These extensive studies indicate quite conclusively that thirty-six is the number of ordinary chromosomes in the somatic cells of both sexes and that the male cells contain one sex-chromosome

while the female cells contain two, making a total of thirty-seven in the male and thirty-eight in the female. These numbers correspond exactly with those of the spermatogonia in the male and the oögonia in the female. This is very significant in relation to our chromosome theory of sex-determination.

VIII. Sex-Chromosomes in Relation to Sex-determination.

It was shown that in the process of spermatogenesis two distinct types of spermatozoa are produced of exactly the same number. The one type contains eighteen ordinary chromosomes plus one sex-chromosome, and the other type contains only the eighteen ordinary chromosomes; this being the result of the unequal primary spermatocyte division, where the eighteen bivalent chromosomes divide and the unpaired sex-chromosome passes over to one pole undivided. In the oögonia there are thirty-six ordinary chromosomes plus two sex-chromosomes. Before the reduction division of the primary oöcytes, in all probability (though this was not actually determined in this animal), all of the chromosomes, including the two sex-chromosomes, pair. This eventually gives rise to ova all of which contain the reduced number of chromosomes or eighteen ordinary chromosomes plus one sex-chromosome.

Since the two types of spermatozoa are produced in equal numbers, fertilization by the one kind or the other is equally possible, and the number of male and female calves born is about equal if a fairly large number of offspring is considered. Sex in the offspring, as determined at the time of fertilization of the ovum by the one or the other type of spermatozoön may be illustrated as follows:

Spermatozoa.	Ova.	Offspring.
$(18 + 1)$	$+ (18 + 1)$	$= (36 + 2)$ = female,
$(18 + 0)$	$+ (18 + 1)$	$= (36 + 1)$ = male.

The results of the above combinations are in exact accord with the number of chromosomes found in the germinal and somatic cells of the two sexes in cattle; and the relation of the sex-chromosomes to sex-determination can not be doubted. All of the five hundred or more so-called theories or rules for con-

trolling sex, including the one in relation to time of service which is commonly practiced by animal breeders, must be abandoned. There is considerable literature on the metabolic theories of sex-determination which will not be discussed here. However, reference may be made to the brief though able discussion of this subject by Babcock and Clausen ('18).

IX. Sex Ratio in Cattle.

It might be well to quote here Pearl's ('17) more recent results of extensive studies on the control of the sex ratio in cattle. He says: "Some earlier statistics appeared to indicate that there was a possibility of influencing the sex ratio by paying attention to this point. It was believed to be of such extreme importance as to justify the careful study of the matter on the basis of much more extended statistics. These statistics we have now collected and analyzed and shall publish as soon as possible. In the meantime it may be reported that, with the more extended statistics in hand, it appears to be conclusively established that there is no definite or permanent relation between the time in the heat period at which the cow is served and the sex of the offspring. The apparent relation between these two factors, which is believed by many breeders to exist and which our earlier statistics appeared to indicate, seems now to be purely accidental, and to have arisen only because of the comparative meagerness of the statistics on which the matter was discussed.

Table II.

Showing the Sex of the Calves following Service at Different Parts of the Heat Period.

Heat Period.	Lapsed Time in Hours from Appearance of Heat to Service.	Sex of Offspring.		Per Cent. of Males.
		Males.	Females.	
Early.....	Under 3 hours	200	192	51.0
Middle.....	Over 3 and under 8 hours	270	252	51.7
Late.....	Over 8 hours.....	187	212	46.9
Totals.....		657	656	50.0

"The summarized results of 1,313 separate and distinct matings given in Table II. will demonstrate this point. In each one of these 1,313 cases the following facts were accurately known, and

reported in such a way that any bias, conscious, or unconscious, of the observer could not have influenced the result: (*a*) the time in hours from the first appearance of heat (œstrum), as noticed by the breeder, to the time the cow was successfully served; (*b*) the sex of the calf resulting from this service.

"It is evident from this table that there is no significant preponderance of females when service is early in heat. There is not now known any method by which the sex ratio or proportion of the sexes in cattle may be effectively controlled by the breeder. A more detailed account of the results, together with further statistics will be published elsewhere."

X. Sex-limited Inheritance in Cattle.

The discovery of the remarkable behavior of certain characters in heredity which can only be plausibly explained by supposing that they are linked with a sex-chromosome or a sex-determining factor still further strengthens our belief in the existence of such a definite factor. Wentworth ('16) reported a case which seems to fall under this general sex-limited group in the inheritance of black-and-white in Ayrshire cattle. While the general breed color is red-and-white, black-and-white animals have been known for some time, as shown by Kuhlman ('15). It is difficult to state whether the black is due to a true black pigment or whether it is simply a very dense red, since chemical solutions have not yet been attempted.

In summarizing the results of the different crosses, Wentworth says, "If the factor of the black-and-white color is represented by B, the hereditary constitutions are as follows: BB is always black-and-white; bb is always red-and-white; Bb is always black-and-white in the male and red-and-white in the female. All of the nine possible matings were discovered, as shown in Table I.

"The expectations here presented are based on the most probable result of each of the matings, considered on an individual basis with reference to the number of animals produced by each type of mating, but without figuring the proportions of the sexes as equal. From these data it would appear that the black-and-white color of Ayrshire cattle behaves in an ordinary

sex-limited manner similar to the horns in sheep as discussed by Wood ('05) and the rudimentary mammæ in swine as reported by Wentworth ('16)."

TABLE I.

RESULTS OF NINE POSSIBLE MATINGS OF AYRSHIRE CATTLE.

Sires.	Dams.	Male Offspring.		Female Offspring.	
		Black-and-white.	Red-and-white.	Black-and-white.	Red-and-white.
BB.....	BB.....	1	0	3	0
BB.....	Bb.....	0	0	0	1
BB.....	bb.....	10	0	0	10
Bb.....	BB.....	3	0	2	1
Bb.....	Bb.....	1	0	1	0
Bb.....	bb.....	4	5	0	4
bb.....	BB.....	0	0	0	3
bb.....	Bb.....	2	1	0	2
bb.....	bb.....	0	7	0	9
Total.....	.....	21	13	6	30
Expected....	.....	20.75	13.25	5.25	30.75

The simple Mendelian scheme of inheritance is quite common in cattle but, to my knowledge, this is the only case of sex-limited inheritance reported in this animal; nevertheless, it is significant, especially in view of our knowledge of the relation between this scheme of inheritance and the sex-chromosomes in many other species.

XI. THE FREE-MARTIN.

The case of the free-martin, the female of two-sexed twins in cattle, is well known to animal breeders to be perfectly sterile although rarely such females are perfectly normal. I do not wish to enter upon a discussion of this subject here for it is a big problem in itself. However, I wish to call the attention of animal husbandmen, who are not in position to keep in touch with all of the zoölogical literature, to the extensive and most painstaking piece of research on the free-martin by Dr. Frank R. Lillie ('17). In this connection I might also call attention to his article on sex-determination and sex-differentiation in mammals (Lillie, '17).

XII. SUMMARY.

1. Thirty-seven chromosomes occur in the spermatogonia. One, the sex-chromosome, is distinctly larger than the others.

2. Nineteen chromosomes appear in the primary spermatocyte division, of which eighteen are bivalent and the other is the unpaired sex-chromosome.

3. In the primary spermatocyte division the heart-shaped sex-chromosome passes undivided to one pole in advance of the other chromosomes.

4. The primary spermatocyte division is evidently the reduction division, giving rise to two different types of secondary spermatocytes, one with the sex-chromosome and the other lacking it.

5. The one type of secondary spermatocyte, which contains the sex-chromosome, gives rise to two spermatids, each containing the sex-chromosome and eighteen ordinary chromosomes.

6. The other type of secondary spermatocyte, which lacks the sex-chromosome, gives rise to two spermatids, each containing only the eighteen ordinary chromosomes.

7. The mature spermatozoa are of two types, equal in numbers. The one type is larger and contains the sex-chromosome. The smaller type is without the sex-chromosome. The larger type is female producing, while the smaller is male producing.

8. Thirty-eight chromosomes occur in the oögonia; two of these are the sex-chromosomes.

9. The reduced number of chromosomes in the female is, in all probability, eighteen ordinary chromosomes and one sex-chromosome which apparently occurs in all of the mature ova.

10. The somatic cells of the male contain thirty-six ordinary chromosomes and one sex-chromosome.

11. The somatic cells of the female contain thirty-six ordinary chromosomes and two sex-chromosomes.

12. The number of chromosomes in the somatic cells of the two sexes is in exact accord with expectations.

13. There is no relation between the time in the heat period at which the cow is served and the sex of the offspring.

14. Sex in cattle, for the present, remains a matter beyond the control of the breeder. It is determined by the sex-chromosomes; it is a matter of inheritance.

15. Sex-limited inheritance strengthens the belief in the chromosome theory of sex-determination.

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EXPLANATION OF PLATES.

PLATE I.

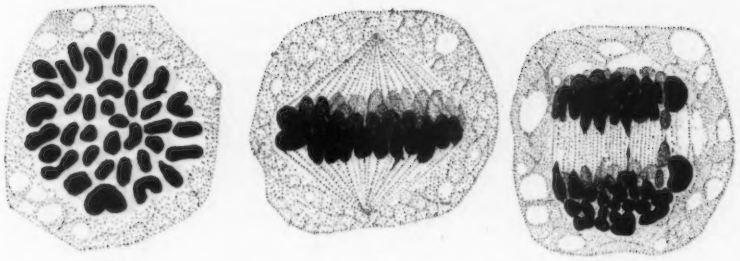
(All of the drawings were made with the aid of a camera lucida. × 2,400, except Figs. 39 and 40 which are × 2,200.)

FIG. 1. Polar view of the metaphase of division in a spermatogonial cell showing thirty-six ordinary chromosomes and the single heart-shaped sex-chromosome.

FIG. 2. Side view of the metaphase of division in a spermatogonial cell showing the thirty-six ordinary chromosomes, and the sex-chromosome at the left.

FIGS. 3 AND 4. Late anaphases of division of spermatogonial cells showing the division of all of the chromosomes, including the large sex-chromosome.

FIGS. 5–9. Late prophase stages of the last spermatogonial cells.



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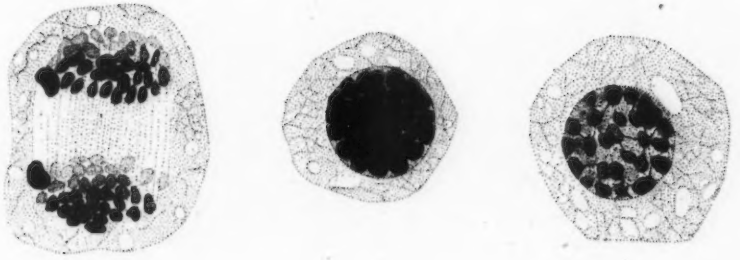

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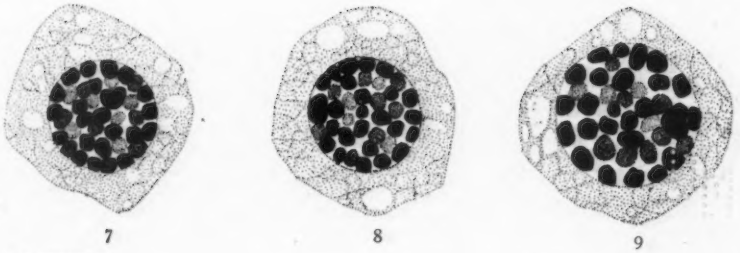

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J. F. WODSEDALEK.

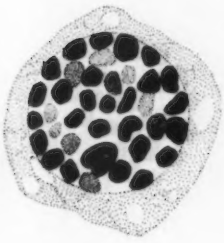
PLATE II.

FIGS. 10 AND 11. Last spermatogonial cells showing thirty-six ordinary chromosomes and the large sex-chromosome.

FIG. 12. Last spermatogonial cell taken from stale tissue showing the persistence of the segregation of the chromosomes in this stage.

FIG. 13. Spireme stage of the primary spermatocyte showing the large sex-chromosome.

FIGS. 14–18. Polar views of metaphase stages of division of the primary spermatocytes showing eighteen bivalent chromosomes and the sex-chromosome which is usually at the periphery of the plate.



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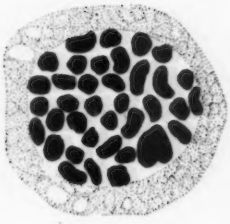

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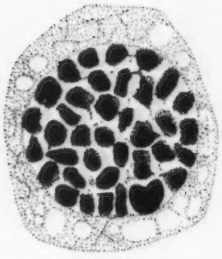

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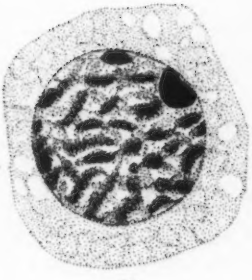

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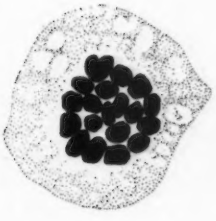

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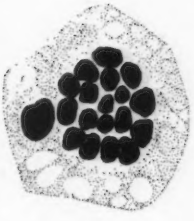

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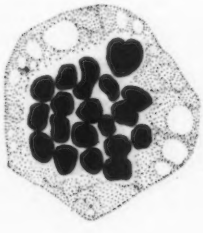

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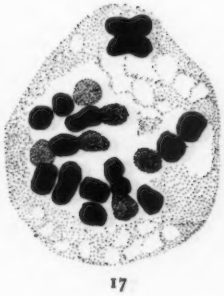

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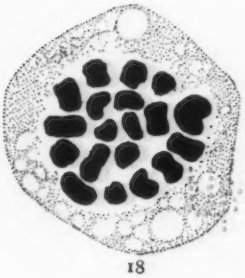

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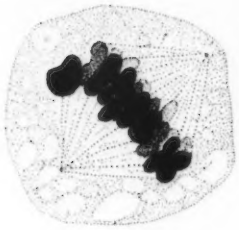
PLATE III.

FIGS. 19–25. Metaphase stages of division of primary spermatocytes showing the passing of the sex-chromosome to one pole in advance of the other chromosomes.

FIGS. 26 AND 27. Late anaphase stages of division of primary spermatocytes showing eighteen chromosomes at one pole and eighteen plus the sex-chromosome at the other.



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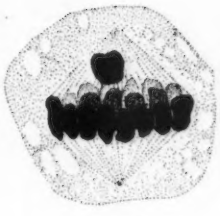

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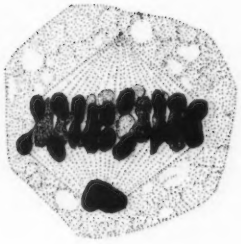

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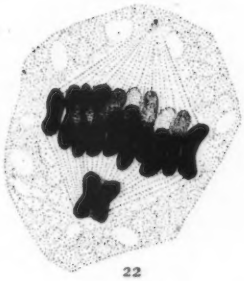

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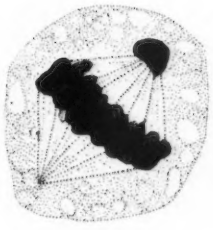

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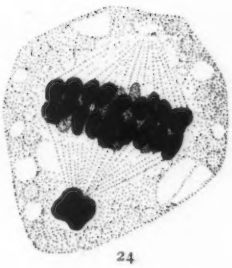

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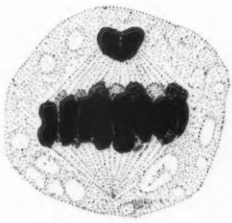

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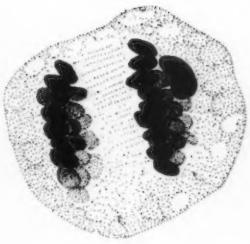

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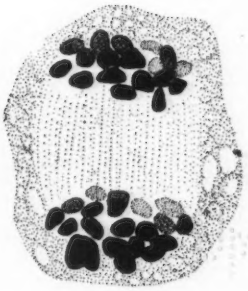

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PLATE IV.

FIGS. 28 AND 29. Cells showing the formation of the secondary spermatocytes from the primary spermatocyte division. The eighteen chromosomes pair loosely to form nine bivalents which are present in the one type of secondary spermatocyte and in the other type are shown the nine bivalents and the sex-chromosome.

FIG. 30. Metaphase stage of division of the one type of secondary spermatocyte showing the nine bivalent chromosomes and the sex-chromosome.

FIG. 31. Metaphase stage of division of the other type of secondary spermatocyte showing only the nine bivalent chromosomes.

FIGS. 32–34. Division stages of the secondary spermatocyte with the sex-chromosome showing the division of all of the chromosomes, including the sex-element, giving rise to spermatids both of which contain the sex-chromosome. When the division of the cell is complete (Fig. 34) each of the nine ordinary chromosome splits into two so that in reality there are eighteen ordinary chromosomes plus the sex-chromosome in this type of spermatid.

FIGS. 35–37. Division stages of the secondary spermatocyte without the sex-chromosome. Figure 37 shows the splitting up of the nine chromosomes at the poles so that there are in reality eighteen chromosomes passed on to the other type of spermatid.

FIG. 38. A newly formed spermatid showing the eighteen chromosomes.

FIG. 39. A mature spermatozoan of the smaller type undoubtedly without the sex-chromosome.

FIG. 40. A mature spermatozoan of the larger type which undoubtedly contains the sex-chromosome.

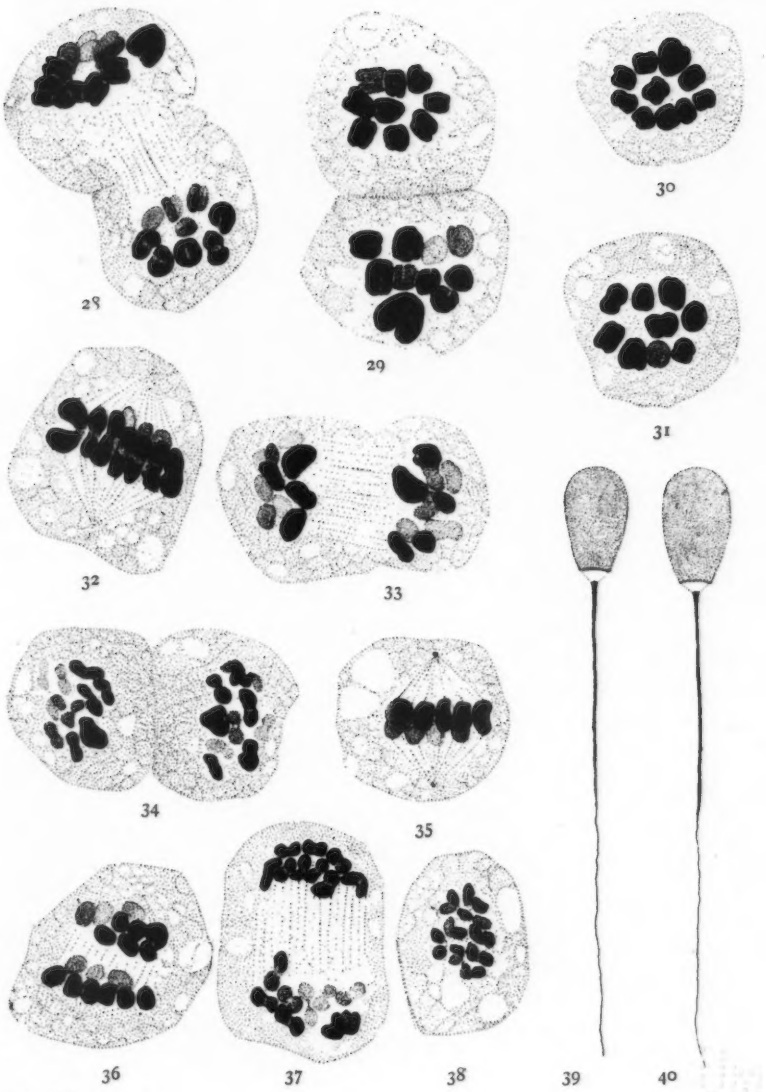

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PLATE V.

FIG. 41. Resting stage of an oögonial cell showing two large nucleoli which are undoubtedly the sex-chromosomes.

FIG. 42. Spireme stage of an oögonial cell showing the two large sex-chromosomes.

FIG. 43. An oögonial cell showing thirty-six newly formed chromosomes and the two large sex-chromosomes.

FIGS. 44 AND 45. Late prophase stages of oögonial cells showing thirty-six ordinary chromosomes and the two sex-chromosomes.

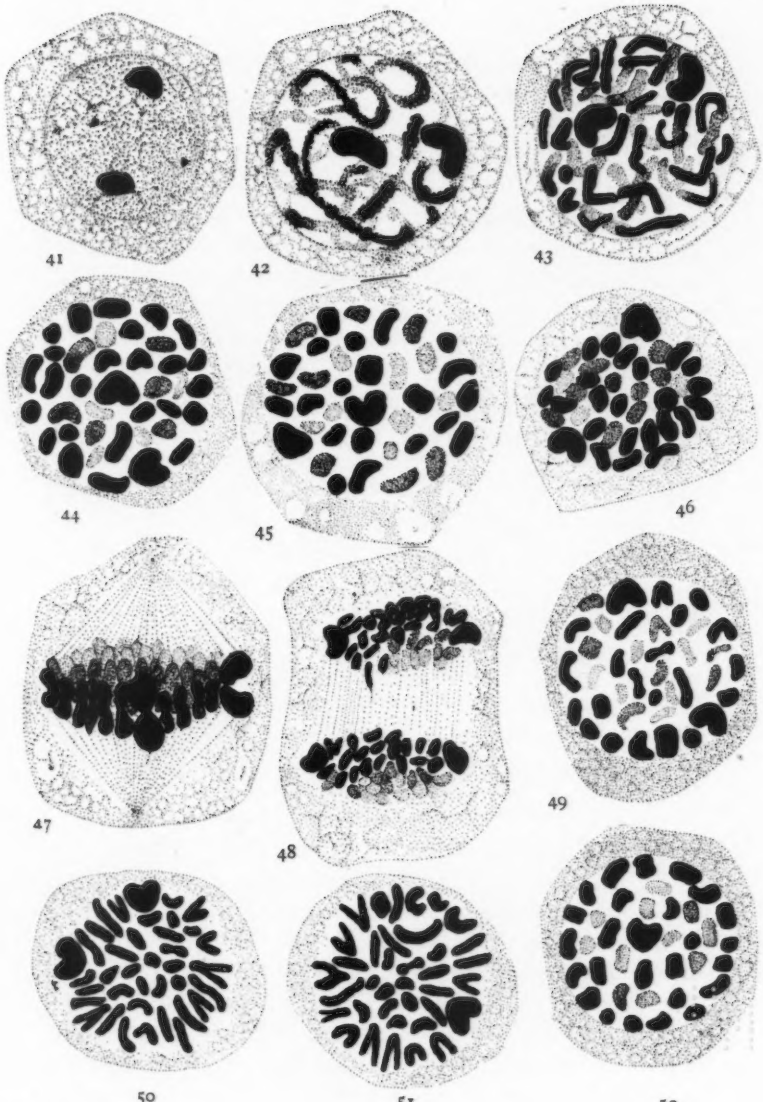
FIGS. 46 AND 47. Metaphase stages of division of the oögonia showing the two sex-chromosomes at the periphery of the plate.

FIG. 49. Liver cell in late prophase stage taken from a female foetus showing thirty-six ordinary chromosomes and the two sex-hromosomes.

FIG. 50. Brain cell in metaphase stage of division taken from a female embryo showing thirty-six ordinary chromosomes and the two sex-chromosomes.

FIG. 51. Brain cell in metaphase stage of division taken from a male embryo showing thirty-six ordinary chromosomes and only one sex-chromosome.

FIG. 52. Liver cell in late prophase stage taken from a male foetus showing thirty-six ordinary chromosomes and only one sex-chromosome.



J. E. WODSEDALEK.

STUDIES IN ARTIFICIAL PARTHENOGENESIS. III. CORTICAL CHANGE AND THE INITIATION OF MATURATION IN THE EGG OF CUMINGIA.[1]

L. V. HEILBRUNN

This study is a record of experiments performed during the summer of 1916 at the Woods Hole Marine Biological Laboratory.

As Morgan pointed out in 1910, the egg of the lamellibranch *Cumingia* is very suitable for study. Like most other eggs it is still immature when shed into the sea-water. Although the first maturation spindle has formed, no polar bodies are thrown off unless the egg is fertilized or treated with the proper reagents. Doubtless some change is necessary before the egg can throw off polar bodies and begin its development.

An effort has been made to determine the nature of this change. Many diverse reagents cause the egg to mature. Although all of these reagents do not occasion the same morphological transformations nevertheless all of them agree in having one specific physical effect on the egg. All release the egg cytoplasm from the restraint of a rigid enveloping membrane. The immature unfertilized egg is surrounded by a stiff vitelline membrane which presses tightly in on it and effectively prevents the throwing off of polar bodies. It is only when the egg is released from this restraint that maturation can proceed.

Physical Make-up of the Egg.

As in *Arbacia*, the *Cumingia* egg is a mass of fluid protoplasm, surrounded by a rigid membrane. Only a few turns of the centrifuge are sufficient to throw to opposite poles of the egg the substances suspended in the cytoplasm. To one pole pass the presumably lighter oil globules, to the opposite pole the heavier pigment. But these suspended particles can go no farther than the poles, for there they are stopped by the vitelline membrane which surrounds the egg. This is a stiff structure and is easily

[1] Contribution from the Zoölogical Laboratory, University of Michigan, New Series, no. 3.

visible under higher power. It is about one micron in thickness. The vitelline membrane must be thought of as the plasma membrane of the egg. As I have pointed out before, students of cellular mechanics have been blind to the fact that egg cells are provided with visible membranes which are plasma-membranes. They have often insisted that no one has ever seen a plasma-membrane. It is easy to prove that the vitelline mem-membrane of the *Cumingia* egg governs osmotic intercourse and is therefore a plasma-membrane.

When *Cumingia* eggs are put into hypertonic solutions they shrink only very slightly. The stiffness of the vitelline plasma-membrane prevents a marked shrinkage. Moreover, a weakly hypertonic solution produces just as much shrinkage as a strong one provided that it does not alter the membrane. On the other hand if the hypertonic solution makes the membrane less rigid by causing it to swell, then the egg shrinks to a great extent. Facts such as these can be interpreted only on the assumption of a stiff plasma-membrane. That this is the vitelline membrane is certain, for there is no other membrane in the vicinity. Certainly there is none inside the vitelline membrane, for when the egg has been left for some time in a hypertonic solution of considerable strength then the coagulated cytoplasm shrinks away from the vitelline membrane and presents a rough uneven surface. Under these conditions it is obviously not surrounded by any membrane.

As to the chemical nature of the plasma-membrane it is essentially protein. It swells in dilute acids or alkalies, and in sodium chloride or sodium iodide solutions. Moreover it does not contain any large admixture of lipoid as can be shown by testing it with Scharlach R solution.

Like many other marine eggs the Cumingia egg is surrounded by a diffuse jelly of the same refractive index as sea-water and therefore invisible. It can easily be demonstrated by various vital stains (*e.g.*, Nile blue sulphate) or by India ink suspensions. This jelly has no apparent effect on the early developmental phenomena. If the eggs are shaken a few times in a test-tube, they are deprived of their jelly. Such eggs react in the same way as those with jelly intact.

As in *Arbacia* the *Cumingia* vitelline membrane is capable of two kinds of cortical change. Both membrane elevation and membrane swelling can occur. If the egg is placed in solutions of low surface tension the tension of the membrane is lowered and it rapidly lifts away from the egg. No doubt the explanation of this process is the same as that I have offered for the similar process in the sea-urchin egg. But in *Cumingia* membrane elevation is not the normal cortical change. When eggs are inseminated the membrane does not become elevated, it becomes swollen.

Treatment of eggs with a reagent which causes either membrane elevation or membrane swelling will result in a throwing off of polar bodies. Thus just as in the sea-urchin egg either swelling or elevation provides the necessary cortical change, although in this egg it is swelling and not elevation which is normal. Finally there is a third way in which maturation can be initiated. This consists in the removal of the membrane.

I shall now proceed to the experimental data considering first membrane elevation, second membrane swelling, and third the removal of the membrane.

Membrane Elevation.

Any substance which markedly lowers the surface tension of sea-water is effective in producing membrane elevation. But if elevation is to be followed by maturation only certain concentrations and certain lengths of exposure can be used in each case. Too high a concentration or too long an exposure generally leads to coagulation. Frequently an over-exposure produces a rupture of the egg membrane, the protoplasm then flows out to form an exovate. Such eggs soon disintegrate.

Numerous reagents were used to produce a lowering of surface tension. Of course the number of successful reagents could have been increased many fold. Thus many of the higher alcohols no doubt act in the same way as the amyl alcohol which I used.

In the experiments the same general proceedure was always employed. In every case, the eggs of only a single female were used. Owing to the fact that the number of eggs obtainable from a single female is comparatively small, I found it best to

perform all of the experiments in low Stender dishes (containing about 50 cc.) instead of in larger fingerbowls. These dishes were very convenient, for they could without any trouble be put on the stage of the microscope. One Stender dish was made to contain the desired reagent and then the eggs were pipetted into it. After varying lengths of exposure the eggs were transferred from the reagent to fresh sea-water also contained in Stender dishes. Then after twenty minutes or more had elapsed the treated eggs were examined.

As the polar bodies of Cumingia are unusually large it is possible to count the percentage of matured eggs under low power and without removing the eggs to a slide. Counts were usually made in this way. Obviously it is not possible to see the polar bodies if the egg is lying with the animal pole down. Hence the per cent. recorded is always too low. Even if all the eggs had polar bodies not many more than fifty per cent. would show them. If the eggs are turned about this difficulty can of course be obviated, but turning is a tedious process and was only occasionally resorted to. Thus the *counts recorded represent minima* and are really only about half as high as they should be. Counts are given in the form of fractions, in which the denominator represents the total number of eggs counted the numerator the number of eggs with polar bodies. For example the fraction 10/50 would indicate that out of 50 eggs counted 10 showed a polar body.

In making up per cent. solutions of volatile liquids, it was found convenient to use 100 c.c. measuring flasks. Thus if a 2 per cent. ether solution was desired 2 c.c. of ether was placed in a 100 c.c. measuring flask and sea-water was added until the solution reached the 100 c.c. mark. Owing to the diminution in volume on mixing the two liquids such a solution is not exactly a 2 per cent. one. However, after the above method of procedure it is easier to calculate the molecular concentration.

The accompanying table gives the results of experiments with eleven substances which lower surface tension. For each substance used it was necessary first to determine the proper concentration. Sometimes this was a simple matter. Thus for saponin almost any concentration is successful. But usually only a narrow range of concentrations will produce the desired

effect. If the reagent is just a little too strong it can not be effectively employed. In many cases only a certain length of exposure is suitable. Too long exposures generally produce exovates. Formation of exovates is represented by the symbol "e" in the table.

With ether and chloroform it is particularly difficult to obtain maturation. In both instances I at first despaired of success. A 3 per cent. solution of ether in sea-water did not produce maturation. With such a solution exovates generally appeared. On the other hand 2.5 per cent. ether had no observable effect on the eggs. With a concentration intermediate between 2.5 per cent. and 3 per cent. success was attained. In the table are given the figures for a representative experiment with ether.

In the case of chloroform the range of effective solutions is even narrower. In making up chloroform solutions very small quantities of the liquid had to be measured out. This was done by counting the drops from a small pipette which was calibrated for the purpose. About 60 drops from this pipette constituted 1 c.c. By placing 8 such drops into a 100 c.c. measuring flask and diluting to the mark a 0.13 per cent. solution was obtained. This solution produced but little effect upon the eggs. Exposures of 3–5 minutes showed only 1–2 per cent. of polar body formation. Thus a 0.13 per cent. solution was apparently too weak. On the other hand, a 0.17 per cent. solution proved too strong. Such a solution was prepared by diluting 15 drops of chloroform (from the pipette mentioned above) up to 100 c.c. with sea-water. A 0.25 per cent. solution was thus obtained and this was then diluted to 0.17 per cent. by adding 5 c.c. of sea-water to 10 c.c. of the solution. The resultant 0.17 per cent. solution was found to be too strong, for it produced exovates. Moreover viscosity tests with the centrifuge showed that it coagulated the egg cytoplasm. Although neither 0.13 per cent. nor 0.17 per cent. chloroform produced maturation, a concentration slightly under 0.17 per cent. did. This solution was made by diluting 10 drops from the pipette up to 100 c.c. Such a solution would ordinarily give 0.17 per cent., but one particular solution was made on an extremely hot day (when the room temperature was 27°). No doubt the drops from the pipette were smaller, owing to the

lower surface tension. Hence the concentration which resulted was slightly under 0.17 per cent. In the table I have referred to this solution as "0.16 per cent." As the figures show, it proved highly effective.

TABLE I.

POLAR BODY FORMATION AFTER TREATMENT WITH SUBSTANCES WHICH LOWER SURFACE TENSION.

Exposure Minutes.	1 Per Cent. Amyl Alcohol.	2.5 Per Cent. Ethyl Acetate.	0.25 Per Cent. Ethyl Butyrate.	0.5 Per Cent. Ethyl Nitrate.	2.8 Per Cent. Ether.	'0.16 Per Cent.' Chloroform.	5 Per Cent. Acetonitrile.	Saturated Solution Phenyl Urethane.	0.2 Per Cent. Chloretone.	Emulsion[1] Toluol.	0.2 Per Cent. Saponin.
1	0		0	0	0		0		29		12
4	50		50	50	100		100		100		50
1	0	0	0	0	0	13	2	0	18	3	20
2	50	50	50	50	100	50	50	100	100	100	50
1	1	0	0	2	1	29	12	0	0	10	33
	50	50	50	50	100	50	50	100	100	100	50
2	12	4	22	14	6	e	e	0	0	e	
	25	50	100	100	100			100	100		
3	12	10	e	e	15	e	e	0	0	e	33
	25	50			100			100	100		50
4	31	13	e	e	22	e	e	0		e	18
	50	50			100			100			50
5		19[2]	e	e	18	e	e	1			20
		50			100			100			50
6	14	12 23[2]	e					2			
	25	50 50						100			
7			e	e	15	e	e			e	19
					100						50
8		23						5			
		50						100			
10	3	16	e	e	5	e	e	9			
	25	50			100			100			
15								43			
								200			

All of the substances mentioned in the table produced a similar morphological change in the egg. All of them caused the vitelline membrane to become lifted away from the cytoplasm. The details of this membrane elevation were watched in a great many different experiments. The membrane first detached itself from the egg at a number of points around the periphery. As a result it appeared slightly thicker and for a minute or two it often

[1] This emulsion was made by stirring 1 c.c. of toluol with 3 c.c. or sea water.

[2] Eggs were shaken while counting. As mentioned above this gives a higher count.

showed small outbulgings which gave it a more or less crenate appearance. Very soon, the membrane lifted away with considerable rapidity. If success was to be obtained the egg had to be removed from the solution at just about the time the crenations appeared.

As in *Arbacia* when the vitelline membrane is lifted from the egg surface, a new membrane, no doubt a precipitation membrane, is immediately formed about the egg cytoplasm. Sometimes it is possible to cause this to become elevated also. Saponin produces the best results. When eggs are exposed for 40 minutes to 0.2 per cent. saponin they are found to be surrounded by two membranes. The outer one of these is quite far from the egg and it was thought at first that it might represent the outer edge of the jelly which had become visible. But this was shown not to be true, for two membranes could be produced about eggs from which the jelly had previously been shaken off.

I have used the eggs of *Cumingia* for a number of years and in some years I have found a tendency for a small per cent. of the eggs to mature without apparently any treatment. In the experiments recorded in this paper careful controls were always kept. In every case at least two hundred eggs of the untreated control were examined for polar body formation. Of the experiments cited in the table, in only one instance did the control show any maturation. In the control for the ethyl nitrate experiment one egg showed a polar body out of considerably over two hundred examined.

The table shows that eleven substances can produce polar body formation and this is in every case preceeded by membrane elevation. These eleven substances differ from each other very widely in chemical constitution. It is almost inconceivable that they should have any one chemical effect in common. Their action must be primarily physical. It is believed to involve a lowering of surface tension. The explanation which I have offered for membrane elevation in *Arbacia* applies equally well for *Cumingia*. For details of this explanation the reader is referred to my earlier papers.

Of course by choosing substances similar to those listed in the table numerous other successful reagents could no doubt be

discovered. Thus it is probable that benzol or xylol would behave as toluol, and that many other alcohols and esters could have been added to those given in the table. Such experiments would scarcely add material for the general argument.

On the other hand it might be thought that the eleven substances in the table represent only a few out of the many that I have tried. It perhaps not infrequently happens that experimenters suppress the record of their failures. But in these experiments practically every substance selected because of its effect on surface tension gave the expected result. There were only three exceptions and in two of these cases I performed only a single experiment with a single concentration of the reagent. Moreover in each of these three cases the reagent employed had some secondary effect on the egg. I shall consider each case in detail.

Ethyl Urethane.—3 per cent. ethyl urethane had practically no effect, although in longer exposures (5–7 minutes) a few eggs with polar bodies were observed. The reagent has some action on the jelly that I have not analyzed. Possibly it is a shrinkage effect. Two minutes after the egg entered the solution it was surrounded by queer looking bubbles. Fifteen minutes later the bubbles had disappeared and their place had apparently been taken by a zone of radiating lines.

Nitromethane.—I used a 5 per cent. solution of nitromethane and exposed the eggs to it for intervals of from $\frac{1}{2}$–10 minutes. No polar bodies were produced as a result of the treatment. Exposure for 4 minutes followed by transfer to normal sea-water resulted in rupture of the vitelline membrane and disintegration of the egg. The eggs left in the nitromethane solution showed a queer transformation. They lost their spherical shape and flattened out into discs resembling huge red blood corpuscles. In addition to this queer effect the reagent also appeared to have some action on the jelly, for some morphological changes were visible around the egg. The nature of these changes I did not stop to investigate.

Acetone.—Two concentrations of acetone were tried. 25 per cent. acetone did not produce membrane elevation when eggs were exposed 1–11 minutes. 50 per cent. acetone produced

membrane elevation, but not polar body formation. Of the eggs exposed ½ minute, 72 per cent. had widely elevated membranes. These eggs, however, could not produce polar bodies for they were thoroughly coagulated. This was shown by a viscosity test with the centrifuge.

Membrane Swelling.

If instead of being lifted off, the vitelline membrane is made to swell, much the same effect is produced on the egg. The increased fluidity of the vitelline membrane results in a lower surface tension.[1] Consequently it no longer exerts as great a pressure upon the egg contents. Thus maturation follows membrane swelling just as it follows membrane elevation.

In order to produce a swelling of the membrane the same reagents were used that were previously found to have been effective for the sea-urchin egg. Evidently the vitelline membranes of both *Cumingia* and *Arbacia* are similar, for they swell under the same conditions.

Sodium Iodide.—Eggs were exposed to 0.6 M sodium iodide. After 15 and 22½ minutes they were removed to sea-water in Stender dishes A and B respectively. Of the eggs in A, 14/100 showed polar bodies. The eggs in B formed no polar bodies. The sodium iodide solution caused membrane swelling.

Hydrochloric Acid.—Eggs were placed in 25 c.c. of sea-water plus 0.7 c.c. *n*/10 HCl. After exposures of ½, 1, 2, 3, 4, 5, 7, 10 minutes the eggs were removed from the acid solution and placed in ordinary sea-water in Stender dishes A–H respectively. In the acidified sea-water the egg membrane swelled and the surface of the egg became sticky. Often the eggs adhered to the bottom of the dish. Counts of eggs with polar bodies gave the following results:

A	½ minute exposure	6/50
B	1	5/50
C	1	10/50
D	3	4/50 (This count was made too early)
E	4	13/50
F	5	15/50
G	7	10/50
H	10	8/50

[1] Many biologists apparently do not understand that solids and pseudo-solids (*i.e.*, gels) exhibit surface tension. This tension is greater for a gel than for the corresponding sol. For references to literature on this subject consult Heilbrunn '15, footnote, p. 166.

The eggs which were allowed to remain in the acid sea-water also formed a few polar-bodies.

Potassium Hydroxide.—Eggs were placed in 40 c.c. of sea-water plus 1 c.c. *n*/10 KOH. In this solution membrane swelling occurred. The eggs formed polar bodies while in the alkaline medium. The first polar body observed was noted after 16 minutes exposure. After 33 minutes, a count gave 14/100 with polar bodies.

Potassium Cyanide.—Eggs were placed in a 0.04 per cent. KCN solution, made by diluting 5 c.c. of 2 per cent. KCN up to 250 c.c. with sea-water. During the experiment the cyanide was not allowed to evaporate, for it was kept in a tightly stoppered weighing-tube. After a 36 minute exposure a count showed 28/100 of the eggs with polar bodies. The potassium cyanide caused membrane swelling probably because of its alkaline reaction.

Hypertonic Sodium Chloride Solution.—In the experiments with acids and alkalies and with sodium iodide the solutions used were approximately isotonic with sea-water. In the course of some other work it was noticed that solutions made by adding $2\frac{1}{2}$ M NaCl to sea-water caused membrane swelling. Hence it was expected that these solutions would also cause polar body formation. Eggs were exposed for 7 minutes to a solution made by adding 5 c.c. of $2\frac{1}{2}$ M NaCl to 25 c.c. of sea-water. As a result 21/100 of the eggs formed polar bodies.

To sum up, in all of these experiments the swelling of the vitelline membrane was in every case followed by the throwing-off of polar bodies in a large percentage of the eggs. The controls of untreated eggs did not form polar bodies. In these experiments with reagents which cause membrane swelling there were no failures. No reagent could be discovered which would produce swelling of the vitelline membrane without at the same time causing the eggs to mature.

Rupture or Removal of the Membrane.

There is a third way in which the Cumingia egg may be freed from the binding pressure of its vitelline membrane. If the eggs are shaken vigorously, oftentimes a certain percentage of the

membranes will be shaken off. Apparently the membrane ruptures at the animal pole of the egg and owing to its elasticity it shrinks away toward the vegetal pole. In many cases its wrinkled remains can be found at this pole. No doubt after the membrane is shaken off a new precipitation membrane forms about the cytoplasm but this is very much less rigid than the original vitelline membrane. The results obtained from shaking *Cumingia* eggs are somewhat variable. The eggs must be shaken sufficiently to rupture or remove the membrane, but they must not be shaken too vigorously or too long. Too much shaking interferes with the mitotic processes underlying maturation and a smaller percentage of polar bodies results. This is in accordance with the observation of Wilson ('01) that shaking prevents cell division.

A number of shaking experiments were performed. In one of the best of these, the eggs were placed in a small 10 c.c. test tube and shaken vigorously by swinging the forearm from a vertical to a horizontal position. They were given 40 such swings in 10 seconds. When these shaken eggs were examined an hour and a half later it was found that 27/56 had polar bodies. Thus practically all of them had matured, for as pointed out in the first part of this paper, the counts represent minima. That the shaking process had actually resulted in a removal of the membrane could be demonstrated in three ways. In the first place the remains of the membrane could often be seen at the vegetal pole. Secondly, when the polar bodies formed they did not appear to be within a stiff membrane as in polar body formation after fertilization. Lastly some shaken eggs were placed in a drop of acetone, of these only 3 out of 50 showed membrane elevation. When normal unshaken eggs were similarly treated with acetone all of them showed an elevated membrane.

Finally there is still another method of freeing the egg from the restraint of its membrane. Although this method produces practically the same results as shaking, the procedure is very different. It was found that in diluted sea-water rupture of the vitelline membrane occurred and as was to be expected, maturation followed. Obviously in diluted sea-water the osmotic pres-

sure forces water to enter the eggs and the membrane bursts. In many instances the ruptured membrane could be seen at one side of the egg. Sometimes exovates were produced. Various dilutions were employed, from pure distilled water to a mixture of 1 part of distilled water to 2 parts of sea-water. In one experiment eggs were exposed to distilled water for 40, 60 and 80 seconds and then returned to sea-water. All three exposures were successful. The 40 second exposure was not counted, the 60 second exposure showed 51/100 polar bodies and the 80 second exposure 52/100 polar bodies. The controls were normal and over 200 eggs which were counted showed no signs of maturation. All the exposures showed some signs of cleavage. One egg reached a stage with about 16 cells. In another experiment eggs were placed into 30 c.c. of sea-water plus 15 c.c. of distilled water. Of these eggs 13/50 showed polar bodies.

The Significance of Cortical Change.

It has been shown that three types of cortical change can be produced in the *Cumingia* egg. All of these free the egg from the restraint of a stiff vitelline membrane. This release from restraint may then be thought of as the essential feature of cortical change and as the direct cause of maturation. When it occurs maturation follows; without it no maturation takes place.

It might be argued that all of the types of cortical change have some other common effect besides the one mentioned. Perhaps they all directly produce an increase of oxidations, which then causes maturation to follow. This would be a difficult relation to conceive of chemically. Moreover there is experimental evidence that maturation does not depend on an increase of oxidations.

The supporters of the oxidation theory of initiation of development have always held that dilute cyanide solutions check oxidations. Thus Loeb, '13, states on p. 26: "It has long been known that the oxidations in the cell can be prevented by the addition of a little potassium cyanide, even when oxygen is present. I have found that the addition of 0.5 c.c. of a 1/20 per cent. KCN solution to 50 c.c. of sea-water is sufficient to stop almost immediately the effect of the spermatozoön in the fertilized sea-urchin egg." Such a solution is 0.0005 per cent.

Some *Cumingia* eggs were placed in a 0.04 per cent. solution of potassium cyanide, five minutes after they had been fertilized. The solution was prepared by diluting 5 c.c. of 2 per cent. KCN up to 250 c.c. with sea-water. Solution and eggs were kept in a glass-stoppered weighing-tube to guard against evaporation of the cyanide. Under these conditions practically all of the fertilized eggs formed the first polar body. Actual count without turning over the eggs, showed 24/50.

On the other hand it might be thought that all types of cortical change produce an increase of permeability. In recent years various observers have claimed that the sea-urchin egg undergoes an increase in permeability either after fertilization or after artificial membrane elevation. These observers have endeavored to show: (1) An increased penetration of dyes, (2) a drop in electrical resistance, (3) a more rapid passage of water into or out of the cell.

When fertilized sea-urchin eggs are placed in dilute solutions of methylene blue, they stain more rapidly than do unfertilized eggs, according to Lyon and Shackell, '10. Runnström, '11, obtained similar results with methylene blue although not with neutral red. The experiments of Lyon and Shackell are frequently cited and are always taken to indicate an increased permeability after fertilization.[1] When unfertilized and fertilized *Cumingia* eggs are placed in dilute solutions of methylene blue or neutral red, the unfertilized eggs take up the dye just as rapidly as do the fertilized eggs.

Fertilized and unfertilized eggs were put into Syracuse dishes containing methylene blue solutions of various strengths. From time to time the eggs were examined over a light and over a dark background, and under the microscope. The color of faintly stained eggs can be much better observed with the naked eye than with the microscope. A more accurate method of determination would involve the use of a colorimeter but the method used was sufficient to show that no marked increase of permeability occurred. The dilutions of the dye were made up from a 0.5 per cent. solution of Grübler's "Methylenblau rectif. nach

[1] They might however indicate nothing more than an increased affinity for dyes on the part of the cytoplasm. Such changes in staining properties are common enough, especially after changes in the colloidal state.

Ehrlich." Five minutes after fertilization some fertilized eggs were placed in dishes A2 to E2 containing 0.1 per cent., 0.05 per cent., 0.025 per cent., 0.0125 per cent., 0.00625 per cent. methylene blue respectively. At the same time some unfertilized eggs were placed in dishes A1 to E1 containing similar concentrations of the dye. After ten minutes had elapsed, the eggs in A1 and A2 were navy blue, those in B1 and B2 pale blue, those in C1 and C2 scarcely colored and those in D1 and D2, E1 and E2 not colored at all. Obviously there was no difference between the two sets of eggs. After fifteen minutes eggs in A1 and A2 were navy blue, those in B1 and B2 light navy blue, those in C1 and C2 light blue, the unfertilized eggs in D1 were a very pale blue, the fertilized eggs in D2 uncolored, in E1 and E2 eggs were still uncolored. The eggs were observed at various times, but no marked changes could be observed. After an hour I thought I might be able to detect a slightly deeper color in the fertilized eggs in A2, B2, C2 than in the unfertilized eggs subjected to the same concentrations of dye in A1, B1, C1, but this was probably due to the fact that the fertilized eggs were slightly more numerous. If entrance of stain is a test of permeability, then certainly there is no sharp difference in the permeability of fertilized and unfertilized *Cumingia* eggs. The concentrations of stain used in the above experiment were not injurious. Even in the most concentrated of the solutions used the eggs proceeded in their development and became motile larvæ. A similar experiment was tried with neutral red. A saturated solution of the stain and 1/2, 1/4, 1/8, 1/16 saturated solutions were used. In no case could any difference in permeability between fertilized and unfertilized eggs be noted.

McClendon, '10, and Gray, '16, have maintained that following fertilization there is a drop in the electrical resistance of the sea-urchin egg. They have interpreted this as indicating an increase of permeability, although of course various other explanations might be given. In the sea-urchin egg the normal process of cortical change is membrane elevation, which would interfere with the experiment. McClendon and Gray therefore were obliged either to wait until the eggs lost their power of undergoing membrane elevation or to so treat the eggs that they lost

this power, before they could make their measurements. In the *Cumingia* egg electrical measurements would be easier inasmuch as the normal cortical change is not membrane elevation.

But the following point should be noted. In any measurements of the electrical resistance of masses of egg cells, it is rather doubtful if one is measuring the resistance of the cells at all. If one conceives of a piled-up mass of spheres resting in a liquid, it is obvious after a moment's consideration that the liquid is broadly continuous from one side of the mass to the other. Thus water flows readily through a pile of shot. If now the spheres are poor conductors as compared to the liquid, and an electric current is sent through the mass, it will flow almost exclusively through the liquid. The resistance then depends on the size and shape of the interspaces between the spheres. In the case of egg cells this may vary in several ways. In the sea-urchin egg it is certain that after fertilization the interspaces between individual eggs in a mass are greater than those before fertilization, for it has been shown (cf. Heilbrunn, '15) that after fertilization the eggs offer much more resistance to compression and hence tend to preserve their spherical shape. Thus after fertilization one might expect the resistance of a mass of eggs to be lower even though the electric current did not pass through the eggs at all.

In the last few years R. S. Lillie ('16, '17) has shown that when fertilized or activated eggs are placed in hypotonic solutions, water enters them more rapidly than it does unfertilized eggs. Similarly in hypertonic solutions (R. S. Lillie, '18) water leaves the fertilized eggs more readily. This is due according to Lillie to an increased permeability of the plasma membrane to water. Lillie's reasoning is a bit difficult to follow. Originally he believed in an increased permeability of the membrane to salts and dissolved substances. This would of course decrease the speed of entrance of water from hypotonic solutions, or the speed of exit to hypertonic solutions, for it would decrease the osmotic pressure upon which the exit or entrance of the water depends. Osmotic pressure is, as everyone knows, dependent upon the impermeability of a membrane to dissolved substances. Increase in permeability to salts would therefore produce the opposite

effect from increased permeability to water. Apparently there is a dilemma.

As a matter of fact it appears to be rather far-fetched to assume a change in permeability to water, since we know the plasma membrane to be at all times permeable to it. R. Lillie's results can be much more simply explained on the basis of my conception of the plasma membrane (see Heilbrunn, '15). Normally before fertilization it is a more or less rigid structure and as such resists the entrance or exit of water from the cell. I showed by measurement ('15, pp. 155–158) that when the membrane was made less rigid as a result of membrane swelling then water left the cell more readily. After fertilization the plasma membrane either itself becomes less rigid, as when membrane swelling occurs, or it is replaced by a less rigid membrane as a result of membrane elevation. Hence water enters and leaves the cell more rapidly.

It is easy enough to decide between R. Lillie's interpretation and mine. If the difference is simply one of relative permeability to water, then in hypotonic or hypertonic solutions the water should enter or leave the fertilized eggs more rapidly, but the final equilibrium point should be the same for both fertilized and unfertilized eggs. However on the basis of my view, not only should the water enter and leave the eggs more rapidly, but the actual equilibrium state should be altered. In hypertonic solutions, more water should leave the fertilized eggs and in hypotonic solutions more water should enter them. In the case of hypotonic solutions Lillie's own figures seem to show that at equilibrium more water has entered the fertilized eggs than the unfertilized.[1]

[1] Lillie's measurements were made on the egg of the sea-urchin *Arbacia*. In this egg the presence of the elevated vitelline membrane or fertilization membrane introduces a complication. As is well known this membrane is a stiff structure. When fertilized sea-urchin eggs are subjected to hypotonic solutions they increase in size rapidly until they reach the elevated membrane. Then further increase in diameter is dependent on the power of the eggs to stretch or rupture the membrane. R. S. Lillie does not state which occurs. As a matter of fact these experiments on endosmosis were done in September when the *Arbacia* season is practically over. At this time the normal membrane elevation is difficult to obtain, and usually, unless the sperm concentration falls within certain very narrow limits, the membrane swells at fertilization. For eggs with swollen membranes the experiment is uncomplicated, as in *Cumingia*.

My experiments with *Cumingia* eggs show clearly that after fertilization not only do the eggs swell more rapidly in hypotonic solutions but their total imbitition of water is greater.

Five minutes after fertilization some eggs were placed in 25 c.c. sea-water plus 15 c.c. distilled water. At the same time some unfertilized eggs were placed in a similar solution. Both sets of eggs were measured after about 45 minutes. By this time the eggs had reached an osmotic equilibrium for a second set of measurements 45 minutes later showed no further change. The results are given in Table II.

TABLE II.

DIAMETERS OF FERTILIZED AND UNFERTILIZED EGGS IN 25 C.C. SEA-WATER + 15 C.C. DISTILLED WATER.

Average Diameter of 12 Unfertilized Eggs in Sea-water 62.65 μ.

Fertilized Eggs After 40–45 Min.	Fertilized Eggs After 85–90 Min.	Unfertilized Eggs After 47–55 Min.	Unfertilized Eggs After 92–100 Min.
μ	μ	μ	μ
70.4	69.2	66.9	67.5
69.2	69.8	66.9	67.5
73.9 × 66.9	68.7	66.9	66.9
71.0 × 68.1	69.2	68.1	66.4
69.8 × 66.9	69.2	66.9	68.1
69.8	71.5 × 63.6	68.1	67.5
69.2 × 68.7	71.5	67.5	66.9
68.1	70.4	66.9	67.5
71.0 × 67.5	69.2	66.4	68.7
70.4	70.4	68.1	68.1
72.7	69.8	68.7	67.5
70.4	70.4 × 68.1	68.1	66.9
69.2			
Average, 69.76	69.53	67.46	67.46

Measurements were made with a Spencer movable scale micrometer at a magnification of about 650 diameters. Not all the fertilized eggs remained spherical. For such eggs the longest and shortest diameters are recorded on the table. In computing the averages the mean of these two measurements was taken. It is obvious from the table that a greater amount of water entered the fertilized eggs. This can not be explained by assuming an increased permeability to water.

It should be noted that the vitelline membranes of the eggs in this experiment did not rupture as a result of being placed in the hypotonic solution. Such a rupture sometimes occurs in solutions of this strength.

In hypertonic solutions fertilized *Cumingia* eggs lose more water than do the unfertilized. The water does not merely leave the eggs more rapidly, more of it passes out and the osmotic equilibrium is different in the two cases. This is shown by an experiment in which fertilized and unfertilized eggs were placed in solutions prepared by adding 1 part of 2 *M* $MgSO_4$ to 2 parts of sea-water. Measurements were made as in the previous experiment. The results are given in Table III.

TABLE III.

DIAMETERS OF FERTILIZED AND UNFERTILIZED EGGS IN 30 C.C. 2M $MgSO_4$ + 60 C.C. SEA-WATER.

Average Diameter of 10 Unfertilized Eggs in Sea-water 61.74 μ.

Fertilized Eggs After 13–23 Min.	Fertilized Eggs After 163–172 Min.	Unfertilized Eggs After 27–33 Min.	Unfertilized Eggs After 143–161 Min.
μ	μ	μ	μ
57.7	51.9 × 58.3	59.4	60.6
53.7 × 58.9	57.7	60.0	60.0
56.5	43.3	59.4 (58.3)	61.2 (56.0)
57.7	42.7	61.7	58.9 (53.7)
57.1 × 58.3	57.7	60.0 (53.7)	60.6 (56.1)
57.1 (51.9)	56.5	59.4	58.9
56.5	57.1	59.4	59.4
59.5 × 54.2	53.7	60.6	57.7
56.0	53.1	61.2	58.9
55.4	49.0	59.4	59.4
	54.8	58.9 × 61.2	59.4
Average, 56.78	52.79	60.05	59.55

The above table requires a little explanation. Some of the eggs, usually the unfertilized ones, became slightly flattened at one pole while in the hypertonic solution. These eggs therefore had a slightly smaller volume than their diameter would indicate. In order to show the extent of the flattening a second measurement was taken from the flattened pole to the opposite pole of the egg. This second measurement is in every case shown in parentheses. Another point also needs explanation. The fertilized eggs measured in the second column had lost so much water in the hypertonic solution that their cytoplasm was coagulated and had in most cases begun to shrink away from the vitelline membrane. This shrinkage was most pronounced in the third and fourth eggs measured and this accounts for the very small size of these eggs.

The table shows clearly that more water leaves the fertilized than the unfertilized eggs. This can not be due to an increased permeability to water for an increase of this sort could produce no such effect. It must be due to a loss in the rigidity of the plasma membrane. In order to make this relation clear, I shall quote from p. 154 of the second paper of this series: "The plasma-membrane of the *Arbacia* egg is a protein gel. As such it possesses a certain degree of rigidity. Suppose a hypothetical system completely surrounded by an extremely rigid semi-permeable membrane. If such a system were placed in a concentrated solution no exosmosis could take place, for if the membrane were perfectly rigid, there could be no removal of solvent from the system without the production of a vacuum. But the membrane would be subjected to a considerable pressure which would tend to make it rearrange its particles in such a fashion that the volume enclosed within it might be lessened. Whereas an extremely rigid membrane would resist such forces one with only a certain degree of rigidity would yield (in the case of sufficient pressure) and exosmosis would be possible. Thus osmosis in an enclosed system depends to some extent at least on the rigidity of the confining membrane. These conclusions apply in some measure to the sea-urchin egg, for the vitelline membrane possesses a slight degree of rigidity." They apply even more directly to the Cumingia egg, for its vitelline membrane, which is also its plasma membrane, is stiffer than that of *Arbacia*. Any loss in the rigidity of this membrane favors either endosmosis or exosmosis. That is why the fertilized eggs of *Cumingia* take up more water from hypotonic solutions and lose more water to hypertonic sloutions than do the unfertilized eggs.

Osmotic change in unfertilized eggs is fairly rapid and no one can deny that the plasma membrane is permeable to water. If then R. S. Lillie's measurements are correct and water enters fertilized eggs more rapidly than unfertilized eggs in hypotonic solutions and leaves them more rapidly in hypertonic solutions than it seems certain that the egg plasma membrane has not markedly increased its permeability to dissolved substances as a result of fertilization. For such an increase in permeability,

by diminishing the osmotic pressure, would slow osmotic interchange and if sufficiently great, would prevent it altogether.

All these points show clearly that the permeability theory of fertilization and artificial parthenogenesis rests on rather doubtful evidence. In *Cumingia* certainly, there are no facts which support it.

Discussion.

Bataillon ('12) in discussing the relations between artificial parthenogenesis in amphibia and sea-urchins, states, "Il n'y a pas une parthénogenèse expérimentale des Oursins et une des Amphibiens. Ce sont des matériaux différents chez lesquels le rythme des cinèses est suspendu et peut être rétabli. S'il y a une Biologie générale, les conditions de l'arrêt ont quelque chose de commun, et les conditions de la mise en branle doivent être comparable." Presumably this is true. The essential factors underlying stimulation to development are very probably alike for every sort of artificial parthenogenesis. It is certain, however, that the subsidiary features of the process are different in each case. It is necessary therefore to study each egg individually, to determine exactly its physical make-up, and to attempt to discover what changes are significant in producing an initiation of development. This is what I tried to do in the case of *Arbacia*.

F. R. Lillie ('19) in referring to my explanation of the process of cortical change in *Arbacia* points out that this explanation "can hardly apply to other cases where the cortical changes present a different morphological form." This is true and I never intended that it should. As a matter of fact, I clearly recognized that even in the one egg there were two distinct types of cortical change which might be produced by spermatozoa. The explanation which F. R. Lillie cites was only advanced to cover one of them. At the same time I offered a different explanation for the other.

F. R. Lillie also objects to my considering cortical change as "a mere epiphenomenon . . . the phenomenon of the primary cortical change is too general to be treated in this fashion and its character in different animal groups is too varied for it to be a mere phenomenon of decrease of surface tension." I must point

out that I have always realized the importance of cortical change. On page 183 of my 1915 paper I stated in a section devoted to the significance of cortical change, that "cortical change, whether it be membrane swelling or elevation, always results in the removal of this obstacle [*i.e.*, a stiff membrane]. The vitelline membrane is either rendered soft by swelling or it is lifted away from the egg surface and its place taken by the no doubt less rigid hyaline layer." I showed moreover that "at least two processes which play a part in normal development would be greatly hindered if some kind of cortical change did not occur."

My results with *Cumingia* fully bear out this point of view. Cortical change in *Cumingia* may take the form of a membrane elevation dependent on a sharp decrease in surface tension. It may more simply be just a membrane swelling. In both cases the result is the same. The egg is freed from an obstacle which impedes development. This in *Cumingia* as in *Arbacia* is the restraining influence of a stiff vitelline membrane. In both eggs the same forces are involved in cortical change. The essential features of the process and the effect on further development are as closely alike as they could possibly be in the two cases.

It should be noted that cortical change in *Cumingia* is not ordinarily followed by segmentation. As in *Arbacia* cell-division in *Cumingia* is preceded by a sharp increase in the viscosity of the cytoplasm. This can be demonstrated by tests with the centrifuge. A number of such tests were made and the relation established beyond a doubt.

Summary.

1. The *Cumingia* egg is surrounded by a stiff vitelline membrane which tightly encloses the fluid cytoplasm.

2. A release from the restraint of this membrane is followed by maturation.

3. Such a release from restraint can be accomplished in three ways; by membrane elevation, by membrane swelling, or by the removal or rupture of the membrane.

4. Substances which themselves have low surface tension produce a lowered surface tension of the membrane and this results in its elevation from the egg surface.

5. Acids, alkalies, and certain salt solutions cause the vitelline membrane to swell.

6. The membrane may be removed from the eggs by shaking, or it may be caused to rupture by immersion in dilute sea-water.

7. All of the above mentioned treatments produce polar-body formation. All of them free the egg from restraint.

8. Maturation in *Cumingia* is not dependent on an increase in oxidations.

9. Cortical change in *Cumingia* produces no increase in permeability either to dissolved substances or to water.

10. The essential features of cortical change in *Cumingia* are the same as those previously shown for Arbacia.

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A CONTRIBUTION TO THE LIFE HISTORY OF *AMŒBA PROTEUS* LEIDY.[1]

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It is only within the last few years that a connected life history of *Amœba proteus* has begun to be formulated. This does not seem strange, even in view of its common use in laboratories, since the difficulties attendant upon the study of the life cycle of such an elusive form, are numerous.

The isolated observations upon the various activities of *Amœba*, the accounts of suppositious appendages, and the descriptions of supposedly new species or varieties, are numerous. The fact that so much observational material upon so many apparently nearly related forms has accumulated, and that so many diverse accounts of the habits of one and the same form have been given, has led some investigators to suspect that perhaps much of this material might be combined to throw light upon the life history of possibly some one or two valid species in whose life cycles some of the forms heretofore described might prove to be merely developmental stages. This suspicion has been strengthened recently by the fact that some of the *Rhizopoda* closely allied to the *Amœba proteus*, whose life cycles are now known, show both widely varying forms and habits during different stages in their growth and reproduction.

Investigations of the life history of *Amœba proteus* which have hitherto been made make it possible to generalize, to some extent, upon the course of some of the changes which take place during its development. There seem to be several modes of reproduction in this form, and several intermediate stages between young and adult before the life cycle is completed. In this communication an attempt is made; first, to review briefly what we know of the life history of *Amœba proteus;* second, to

[1] Grateful acknowledgment is here made of the aid of Professor H. D. Reed, of the department of zoölogy, Cornell University.

describe an apparently hitherto unknown method of reproduction; and third to record some observations upon the development and growth of the forms produced by this new method.

The first observer to suggest that another method of reproduction other than simple fission might obtain among the *Amœba* was Varter ('56) who recorded the presence of numerous fine granules which he observed filling the bodies of some *Amœba radiosa*. These granules he looked upon as fragments of the nucleus, and since, when they escaped from the body, they moved with a curious jerky motion, he inferred that they must be provided with flagella, and termed them spermatozoids. The observations extended no farther. Again in 1863 he observed the granulation of the nucleus in *Amœba princeps*, noting that the original nucleus divided several times and gave rise to many smaller nuclei, which he termed, though apparently with meager foundation, reproductive cells.

Wallich ('63) noted that when *Amœba villosa* "died," the nucleus, which had divided several times, escaped from the body surrounded by bits of protoplasm, of globular form. He observes that the fate of these globules is unknown to him, but believing that they might function in some reproductive process, termed them scaroblasts. Referring to the many different species of *Amœba* which appeared in his cultures, he writes (with reference particularly to *Amœba princeps*, *diffluens*, and *radiosa*): "It will, I think, eventually be found that these are mere transitory phases of one and the same species." Later, he observes with greater conviction: ". . . though not prepared to affirm that the whole of the varieties of *Amœbæ* are reducible to a single, primary, specific type, I candidly confess that the balance of the evidence appears to me to point to such a conclusion, and to indicate that the divergence in the form and outward characters may be wholly dependent on the local and even temporary conditions of the medium in which the young animal happens to make its appearance in the world."

It is to Scheel ('99) that we owe our first complete knowledge of a type of reproduction different from the familiar method of binary fission. In 1899 he described a process of reproduction in *Amœba proteus* which he termed *schizogony*, after a somewhat

similar mode of multiplication occurring among the *Sporozoa*. As it occurs in *Amœba proteus* its course is as follows: The individual encysts; the nucleus divides into several smaller nuclei; these migrate to the periphery of the cell; the protoplasm of the cell divides into as many equal portions as there are nuclei; the cyst wall ruptures; and the nucleus-containing bits of protoplasm emerge, each a complete *Amœba* and an epitome of its parent. In analogy with the spores of the *Sporozoa* these were given the name, pseudopodiospores. More recently Calkins ('05) has noted a method of reproduction in which the nucleus produced by repeated division, gametic nuclei, whose fusion resulted in other nuclei which became the nuclei of the smaller individuals after the manner of the pseudopodiospores just mentioned. The young *Amœbæ* soon assumed the form hitherto called *Amœba radiosa;* passed through this stage and became the common *Amœba proteus*.

Another apparent mode of reproduction was observed by Metcalf ('10). In this method the observer is of the opinion that the sequence of events is: Mature individuals produce globular masses of protoplasm, which are termed gemmules. These develop flagella and assume a cercomonadoid form; later they fuse by two and two, lose the flagella, and develop into adult *Amœbæ*. It is stated that possibly the life cycle of *Amœba proteus* may require a year or more for its completion, and may exhibit during its course three, or even more, modes of reproduction.

Schepotieff ('08, '09) states: "All of these examples suggest that in the case of the *Amœba* [*proteus*] the developmental cycle may be completed in very different ways."

It has been shown, as we have said, that *Amœba radiosa*, formerly regarded as a distinct species, is merely a stage in the development of *Amœba proteus*. It may be that this also is true of such recognized species of *Amœba* as *villosa*, *princeps*, *diffluens*, etc. It is suggested elsewhere in this paper that the species known as *Amœba guttula* is but a developmental stage in the cycle of *proteus*. Vahlkampf ('05) has shown that *Amœba limax*, at least, is to be regarded as a distinct species.

Preparation of Cultures.

The material from which the *Amœbæ* herein described were reared was obtained from a pool in a cattail marsh, in about two feet of water among decaying lily pads and *ceratophyllum*. The thin glutinous deposit upon the bottom and especially on the lily pads which had fallen to the bottom was found to be rich in *Amœba proteus*. In the laboratory the material was distributed into several battery jar aquaria after having been filtered through cheese cloth to remove the larger creatures.

An immediate examination of the material showed that beside the active *proteus*, *radiosa*, *guttula*, and *limax*, there were many encysted protozoa. One encysted form which appeared in large numbers, and which confusingly resembles some of the smaller encysted *Amœbæ* was *Vorticella microstoma* (Fig. 4). Under ordinary magnification its distinguishing feature, the crescentric nucleus, is not visible. With the 1.8 mm. objective it becomes faintly discernible, but it is best seen, and the cyst is indubitably identified apparently only when stained. Methyl green and iodine gave the best results.[1]

After some weeks had elapsed *proteus* and *radiosa* appeared in large numbers. The material was now transferred to a dozen small petri dishes and kept in a constant temperature of about 75 degrees Fahr. After a space of a fortnight there was begun the transfer of inoculation of *Amœba proteus* to 4 cm. stender dishes, furnished with straw infusion or oak leaf infusion, and free from all protozoan forms. The infusions were prepared by boiling the straw or leaves for several hours, and decanting off the dark brown liquor, to be diluted to the optimum strength. A slimy scum formed upon the surface of the infusions after a few days time, which when stirred up and caused to sink to the bottom furnished a nutritive substance upon which the *Amœbæ* throve.

From the stender dishes individual *Amœbæ* were removed from time to time and kept in shallow cells sunk in slides of unusual thickness. The slides employed were furnished with a device

[1] Methyl green stain: Saturated alcoholic solution methyl green, 3 parts; 2 per cent. aqueous solution acetic acid, 1 part; water 3 parts. Iodine stain: saturated alcoholic solution iodine, 1 part; water, 2 parts. For various protozoan stains, see Hausman, "Fresh Water and Marine Gymnostominan Infusoria" (in press).

for supplying water to take the place of that carried off by evaporation (Fig. 3).

The method of removing individual *Amœbæ* from stender dishes was as follows, and can be used with success for the isolation of any of the larger protozoan forms: A drop of water containing the *Amœbæ* was placed upon an ordinary slide, and, uncovered, searched with the 16 mm. objective and 8 × eyepiece. When *Amœbæ* were located they were removed by means of what is termed an isolation pipette (Fig. 2). A long rubber tube attached at one end to a glass tube drawn out to a very fine tip, and at the other to a small compression bulb, enables one to select and withdraw very minute objects with considerable precision. Both the stender dishes and growing slides were kept at a temperature of 80 degrees Fahr. in a large aquarium jar heated by a carbon filament lamp placed in the bottom, the current being controlled by a rheostat. By means of this simple device unvarying temperatures could be maintained for any desired length of time. The advantage of such a culture oven is that light is admitted freely on all sides (Fig. 1).

Formation of Apseudopodiospores.

One of the stender dishes which had been inoculated with adult *Amœba proteus* proved very productive, the individuals increasing rapidly, apparently by means of binary fission, since many were observed in process of division. The bodies of the largest individuals became filled with minute bodies, which upon staining seemed to be nuclei. The individuals bearing these were extremely sluggish. The pseudopodia were short; exhibited very little movement, changed their shape but slowly, and upon the functional posterior of the body absent altogether (Fig. 5).

After an interval of four days had elapsed a reexamination of some of the sediment from the same stender dish was made and it was found that the numbers of the large *proteus* had appreciably decreased and that their places were taken by a multitude of very small amœboid forms, of an average diameter of 4 or 5 microns. The majority of these exhibited feeble movements of an amœboid kind. Some were globular, some possessed an irregular body outline, though definite pseudopodia were lacking.

(Fig. 10). These, I was later led to believe, originated from the bodies of the larger multinucleated *proteus*. Several of these latter individuals while under observation were observed to give rise to smaller individuals of the form already described. The process was as follows: the animal, which had been moving slowly and apparently without much vigor, gradually came to rest with the pseudopodia upon the then functional anterior portion of the body lobate with slightly pointed tips (Fig. 6). The posterior portion became semi-globular and towards this the greater number of the minute suppositive nuclei within the body plasm migrated. After an interval of about seven to ten minutes the ectoplasm surrounding this globular posterior extremity appeared to disintegrate and from the interior there floated forth several hyaline globules about 4 or 5 microns in diameter. These were followed, after a few minutes by several others, and then a constant outflow began that continued until upwards of thirty of the hyaline spheroids had been extruded (Fig. 7). These were apparently identical with the forms which had made their appearance in the culture. Since these are judged not to be fundamentally different from the pseudopodiospores, of Scheel, but since they exhibited no lobose pseudopodia, they are here called for convenience, *apseudopodiospores*. Some of these, immediately after extrusion began to move slowly, bulging the very thin ectoplasm at several points, yet without forming any definite pseudopodia. Others, synchronous with their emergence, disintegrated. Still others floated away without exhibiting any signs of motion (Fig. 8). It may be that these were gametic forms, and fused before further development.

Towards the time of the completion of apseudopodiospore ejection, the parent *Amœbæ* usually gave signs of renewed activity, elongated perceptibly, and then began apparently to make efforts to move away (Fig. 9). However, after an interval of from twenty minutes to half an hour, they disintegrated. Some disintegrated at once, leaving the apseudopodiospores behind, but usually they were extruded from the globular posterior portion of the animal, the anterior part retaining its integrity. It appeared as though not all of the minute nuclei were used up at the time of the production of one "litter" of apseudopodio-

spores, and that some of them (those in the anterior portion) were lost.

M. Popoff ('11) has recorded a type of reproduction in *Amœba minuta*, a marine species, similar to the one described above, with the difference, however, that the resulting spores were gametes, which later fused. Schmidt ('13) likewise, described this same sort of reproductive activity as occurring in another marine species, *Amœba aquitalis*.

The apseudopodiospores however, at least the majority whose development was watched, were not gametes.

The smallest of the young *Amœbæ* (as we shall call them) those which have just been separated from the parent body are about 3 to 5 microns in diameter and are extremely sluggish. The body is sub-globular and changes its outline but little during the very slow movement. No pseudopodia are developed. The protoplasm is clear, and contains a few small, angular, transparent granules. No contractile vacuole could be seen, nor where the creatures observed to feed (Fig. 10).

With growth comes an increase in activity and a progressively greater irregularity of the body outline (Fig. 11), until at length true lobate pseudopodia make their appearance (Fig. 12). The number of granules within the body increases, food is taken by engulfing, and the protoplasm assumes a grayish hue. This color may be due both to the number of particles within the endoplasm and to the augmentation of its volume.

At the time of the appearance of the true pseudopodia the body is unsymmetrical, but as growth proceeds are more or less radiate arrangement of the pseudopodia takes place, at first not well defined, but becoming more and more pronounced with the increase in size (Fig. 13).

The pseudopodia now become more extended, and tend to develop more acuminate tips. With increasing length and sharpness the pseudopods seem to become more rigid, and spine like, and the granules migrate from them into the more globular central mass of the body, leaving them clear (Fig. 14).

During the time when the young *Amœbæ* are passing from the apseudopodia stage to the *radiosa* stage they confusingly resemble, if indeed they are not exactly similar to the species

known as *Amœba guttula* (Fig. 11), and it is suggested that possibly this creature hitherto accorded specific rank, may be merely a developmental stage in the cycle of *proteus*. That *Amœba radiosa* was named from the *radiosa* stage of *Amœba proteus* has been indicated. The *radiosa* stage which we have observed in this developmental series may be similar.

A new type of modification now takes place, as has been said, when the pseudopodia become longer and more spinous. During this stage, in respect to size, configuration, and characteristic spineous, immobile, hyaline, ray-like pseudopodia, the creatures are apparently indistinguishable from the species known as *Dactylosphærium radiosum*. Hence we shall term this stage the *Dactylosphærium* stage (Fig. 15).

The genus *Dactylosphærium* was established by Hertwig, and Lesser ('74) to receive the organism which they described as *Dactylosphærium vitreum*. The species now known as *Dactylosphærium radiosum*, however, was not referred originally to that genus, but to the genus *Amœba*, as *Amœba radiosa*, by Ehrenberg ('30). It was transferred to the genus *Dactylosphærium* by Bütschli ('80), who however erroneously called it *Dactylosphæria*. Cash ('05) says of it: "The body consists of granular protoplasm and when all the pseudopodia are withdrawn it may become spherical or bluntly lobed; or it may assume an active amœboid phase, when it is hardly, *if at all*,[1] to be distinguished from the smaller forms of *Amœba proteus*." It occurs in marshes and pools, "less common than *Amœba proteus, with which it is usually associated*." [2]

It was observed that not all of the young *Amœbæ* acquired this *Dactylosphærium*-like form. Some became small *radiosa*, passed on to large *radiosa*, and thence on to the *proteus* form. It seemed to be the usual thing, however, for the majority to assume the form of *Dactylosphærium*, and the suggestion is made that perhaps *Dactylosphærium radiosum*, like other former species, may be forced to relinquish its specific distinction.

From the bodies of those individuals which had assumed the *Dactylosphærium* form, lobate pseudopodia were occasionally protruded, and at length the creature became almost globular, and

[2] The italics are the author's.

then proceeded to take on a form similar to the adult *proteus*. Such individuals were isolated, and soon grew in size to adult form, and were, apparently, *Amœba proteus*. Fission was observed to take place among some of these, but no further reproductive activities were noted. The entire sequence of events which have been enumerated took place within three months, the cultures being kept in the glass oven already described, at an average temperature of 80 degrees Fahr.

Some of the individuals of nearly adult proportions developed long, whip-like, and flexible pseudopodia, often more like long threads (Fig. 16). These were clear, even the stouter ones being devoid of granules. Gruber ('11) reported a similar type of pseudopodium (there was but one in this case) which was sent out from the body and moved about as if an organ of exploration.

Some of the most bizarre forms occur during the transition period between the smallest form and the *Dactylosphærium* stage. Fig. 14 represents forms, all but one of which were taken from the same slide.

In Fig. 18 an attempt has been made to group the various forms observed in a tentative cycle of development. The nine arbitrary stages appear to occur in the following order:

1. Adult stage.
2. Division of the nucleus, and migration of the nuclei to the posterior extremity of the body.
3. Escape of apseudopodiospores.
4. *Amœba guttula* stage.
5. Small *Amœba proteus* stage.
6. *Amœba radiosa* stage.
7. *Dactylosphærium radiosum* stage.
8. Resumption of amœboid form.
9. Growth to adult size.

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PLATE I.

FIG. 1. Aquarium jar culture oven. *a*, thermometer; *b*, cardboard cover; *c*, aquarium jar; *d*, cultures; *e*, iron tripod; *f*, lamp; *g*, copper wire rack for lamp.

FIG. 2. Isolation pipette.

FIG. 3. Growing slide. *a*, sponge, for absorbing excess water; *b*, cover glass; *c*, glass tube carrying thread, as siphon; *d*, lower half of glass vial, cemented to the slide with balsam, as reservoir.

FIG. 4. *Vorticella microstoma*, encysted.

FIG. 5. Unusually large *Amœba proteus*, with body filled with minute nuclei.

FIG. 6. *Proteus*, resting before extrusion of the apseudopodiospores.

FIG. 7. Extrusion of the apseudopodiospores.

FIG. 8. Immobile, globular apseudopodiospores.

FIG. 9. *Proteus* after extrusion of the apseudopodiospores.

FIG. 10. Active apseudopodiospores.

FIG. 11. Apseudopodiospores during the stage when they resemble *Amœba guttula*.

FIG. 12. Small *proteus* stage.

FIG. 13. *Amœba radiosa* stage.

FIG. 14. Apparent transitional forms between the *radiosa* and the *Dactylosphærium* stages.

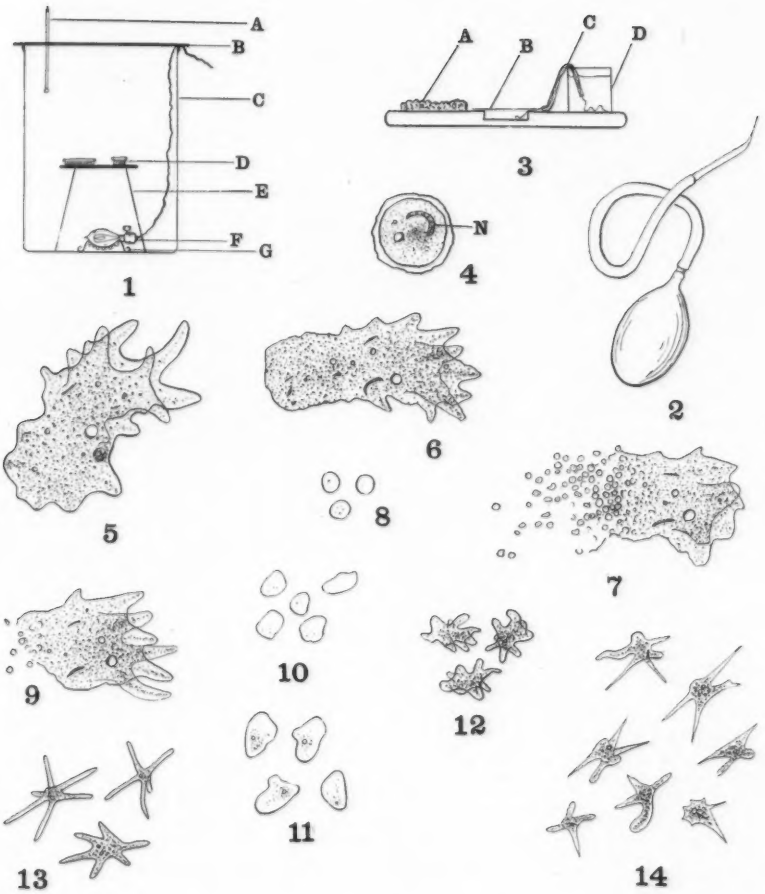
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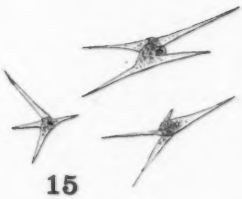
PLATE II.

FIG. 15. *Dactylosphærium radiosum* stage.

FIG. 16. *Amœba proteus* in the adult stage, exhibiting long attenuated pseudopodia.

FIG. 17. Adult normal *Amœba proteus* (stained).

FIG. 18. Tentative Cycle of Development. 1. Adult stage. 2. Division of nucleus; migration of nuclei to posterior. 3. Extrusion of the apseudopodiospores. 4. *Amœba guttula* stage. 5. Small *Amœba proteus* stage. 6. *Amœba radiosa* stage. 7. *Dactylosphærium radiosum* stage. 8. Resumption of amœboid form; retraction of pseudopodia. 9. Growth to adult.



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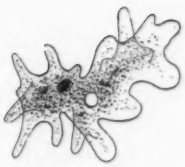

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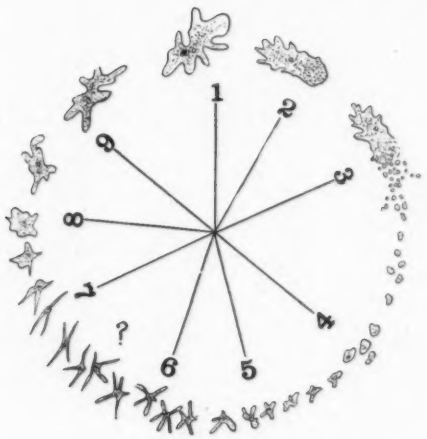

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